CHARACTERIZATION OF *AGROBACTERIUM TUMEFACIENS* ADSORPTION TO POTATO TISSUE

BY

DANIEL ALBERT KLUEPFEL

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1984

ACKNOWLEDGEMENTS

The work completed here at the University of Florida was aided greatly by the support and guidance of many people. Heading the list to receive thanks is my committee chairman Dr. Steven G. Pueppke. His seemingly boundless patience, accurate guidance, and constant availability resulted in the cultivation and maturation of my ability to develop a truly scholarly approach to the art of scientific investigation. I thank you. I am also indebted for the help given by Dr. H. Aldrich, Dr. G. Erdos, and especially Dr. H. Berg in distinguishing fact from fiction when peering through the electron microscope. The time spent by John Ransdell and Steve Linda sharing their expertise with me is greatly appreciated. I also appreciate the critiques given my dissertation by the other members of my committee Dr. R. Stall, Dr. D. Mitchell, Dr. B. Gurley, and Dr. B. Moudgil. Finally, innumerable thanks go to my wife, Marjan, for her help with the statistical analysis, valuable discussions of the data, love and understanding. This work would not have been possible without the financial support of the University of Florida.

TABLE OF CONTENTS

PAGE

ACKNOWLEDGEMENTS ii

ABSTRACT v

CHAPTER ONE INTRODUCTION 1

CHAPTER TWO LITERATURE REVIEW 3
General Adsorption Chemistry 3
Adsorption of Bacterial Invadors 8
Adsorption of Rhizobium spp. to Root Surfaces 10
Adsorption of Agrobacterium tumefaciens to Plant Surfaces 14

CHAPTER THREE ADSORPTION OF AGROBACTERIUM TUMEFACIENS TO POTATO (SOLANUM TUBEROSUM) TISSUE 31
Introduction 31
Materials and Methods 33
Results 35
Discussion 44

CHAPTER FOUR THERMODYNAMICS OF AGROBACTERIUM TUMEFACIENS ADSORPTION TO PLANT SURFACES 49
Introduction 49
Materials and Methods 50
Results 53
Discussion 61

CHAPTER FIVE EFFECT OF AGENTS THAT ALTER IONIC AND HYDROPHOBIC INTERACTIONS ON AGROBACTERIUM TUMEFACIENS ADSORPTION 67
Introduction 67
Materials and Methods 68
Results 76
Discussion 87

CHAPTER SIX SCANNING ELECTRON MICROSCOPY OF POTATO TUBER TISSUE INOCULATED WITH AGROBACTERIUM TUMEFACIENS 93
Introduction 93
Materials and Methods 94
Results 96
Discussion 113

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment
of the Requirements for the Degree of Doctor of Philosophy

CHARACTERIZATION OF AGROBACTERIUM TUMEFACIENS ADSORPTION TO POTATO TISSUE

By

Daniel Albert Kluepfel

August 1984

Chairman: Steven G. Pueppke
Major Department: Plant Pathology

The Freudlich adsorption isotherm accurately defines the adsorption of Agrobacterium tumefaciens to potato tuber discs. This isotherm describes a system where the available binding sites are not saturated. The A. tumefaciens isotherm was developed for inoculum densities that ranged from 10^1 to 10^9 bacteria/ml of buffer. Adsorption is rapid with more than 2.8×10^4 bacteria adsorbing per potato disc within 0.5 min. Agrobacterium tumefaciens dissociates from the disc and reaches equilibrium within 3 to 4 hr at 6°C. The adsorption isotherm was examined over a more limited initial inoculum density at three different temperatures: 6°C, 28°C, 38°C. Analysis of these isotherm data with the Clausius-Clapeyron equation shows that the heat of adsorption is close to zero. These combined data suggest that the adsorption of A. tumefaciens to potato tuber tissue is governed by physical adsorption. Optimum adsorption of A. tumefaciens to potato tissue occurred at pH 7.2, and adsorption was reduced at either pH 4.5 or 9.5. Adsorption to hydrophobic

plastic was insensitive to changes in pH. Conversely, the adsorption to hydrophilic plastic surfaces was optimum at pH 4.5 and decreased with increasing pH. The isoelectric point of A. tumefaciens suspended in water is approximately pH 2.9, as determined by zeta potential measurements. Adsorption of A. tumefaciens cells to potato tissue was greatly reduced by detergents at concentrations as low as 0.05% (v/v). Anionic, cationic and neutral detergents all were effective. The effect of agents that influence both hydrophobic and ionic forces is consistent with the proposed physisorption mechanism for A. tumefaciens. Calcium chloride treatment of the bacteria or EGTA treatment of the potato tuber discs did not, however, significantly affect bacterial adsorption. When viewed with the scanning electron microscope, bacterial colonization of the potato surface was the same for both the virulent A. tumefaciens strain ACH5 and its heat-cured derivative ACH5C3. The only ultrastructural feature that distinguished tissues inoculated with the virulent strain from those inoculated with the avirulent strain was the appearance of tumors five days after inoculation with the former.

CHAPTER ONE
INTRODUCTION

Microorganisms readily accumulate on both natural and man-made structures. They quickly colonize structures in aqueous environments and the surfaces of both plant and animal cells. There is evidence that in some mammalian systems adsorption of bacteria to the host cell surface is an important factor in pathogenicity (Arbuthnott and Smyth 1979, Berkeley et al. 1980, Bitton and Marshall 1980, Ofek and Beachey 1980). The importance of adsorption in plant-bacterial interactions also has been receiving considerable attention (Dazzo 1980a, 1980b, Pueppke 1984a, 1984b).

The surfaces of most plants are colonized by a large number and variety of bacteria (Blakeman 1982, Foster and Bowen 1982, Suslow 1982). The presence of these bacteria, however, rarely leads to necrotic plant disease. Even more unusual is the establishment of a beneficial symbiotic relationship. However, when plant disease or beneficial symbiotic interactions do occur, the role of bacterial adsorption is not clear.

Bacteria have the ability to adsorb to plant, animal, and inanimate surfaces quite tenaciously and, at times, in very large numbers. This phenomenon is of major ecological significance for bacteria. Microbial adsorption and colonization of surfaces provide bacteria with a favorable ecological niche where nutrient levels are high and protection occurs against unfavorable environmental factors (Bitton and Marshall 1980, Costerton et al.

1981). The following literature review will examine some of the features associated with bacterial adsorption to solid surfaces. Salient examples from plant-bacterial systems will be discussed in terms of specificity, adsorption mechanisms, and the relationship between adsorption and the pathogenic or symbiotic responses.

CHAPTER TWO
LITERATURE REVIEW

General Adsorption Chemistry

A colloid is a suspension that is neither a true solution nor a complete two phase system. Colloidal particles are small enough to remain widely dispersed and in constant motion due to bombardment by solvent molecules. Particles in collodial systems characteristically remain suspended and settle very slowly, whereas they sediment very rapidly under centrifugal force. Colloids also cause light to scatter, a useful tool for particle size and concentration determinations. The stability of a colloid, i.e. the tendency of the particles to remain in a highly dispersed state, is partially governed by the electrical charge carried by the particles. Particles within a colloid often contain a large homogeneous charge. This charge causes the particles to repel one another, thereby helping to maintain the stability of the colloid (Saunders 1971, Lips and Jessup 1979).

Bacteria fall within the size range of colloid particles and often exhibit the properties of a colloidal suspension (Marshall 1976). The adsorption of bacteria to solid surfaces involves many of the same forces of attraction and repulsion that operate in the adsorption of inert colloidal particles to solid surfaces (Daniels 1972, Marshall 1976, Berkeley et al. 1980, Bitton and Marshall 1980). Consequently, bacterial suspensions can be considered to be living colloidal systems. Because the bacterial surface is highly charged, the use of

physical (nonbiological) colloidal adsorption systems as models can provide useful information concerning bacterial adsorption. However, it is important to remember that bacteria are living organisms capable of movement and altered activity in response to physiological stimuli.

There are two basic types of adsorption of particles from a liquid phase onto a solid. These two processes, termed physisorption and chemisorption, differ in several fundamental respects (Brunauer 1943, Saunders 1971, Tompkins 1978). The most basic difference is the type of molecular force mediating the adsorption process. Van der Waals forces are primarily responsible for mediating physisorption. Chemisorption requires chemical reactions, i.e. formation of covalent bonds. Chemical bonds in chemisorption systems are much stronger than the forces that account for physisorption. The heat of adsorption, a measure of the bond strength, is on the order of 10 kcal in physisorption and greater than 20 kcal in chemisorption. The basic difference in the bond type is also thought to influence the specificity of adsorption. At low temperatures van der Waals mediated adsorption occurs between any two competent surfaces, but chemisorption depends on the affinity of the two adsorbing components for each other.

The rate of adsorption also is a useful indication of the adsorption type. The rate of physisorption is extremely rapid, whereas chemisorption requires energy of activation for bond formation and, in general, is much slower. In some nonbiological systems, however, the heat of activation can be extremely small, and thus chemisorption can occur relatively rapidly. Physisorption onto a solid surface may be multilayered or monolayered, but chemisorption always occurs in a monolayer. The number of particles adsorbed in a physisorption system is a function of the particle concentration. In chemisorption the

adsorption is a function of both the area of the solid surface and particle concentration. A final contrasting feature is that of desorption. The desorption of particles occurs readily in physisorption, but chemisorbed particles are only removed with some difficulty.

A useful method of adsorption data analysis is the development of an adsorption isotherm. Adsorption isotherms are plots of the number of particles bound versus the number of particles remaining in the suspension at equilibrium. There are four or five basic types of adsorption isotherms (Brunauer 1943, Tompkins 1978). Although adsorption isotherms do not provide data on adsorption mechanisms, other analyses can be used to differentiate chemisorption from physisorption. Analysis of adsorption at various temperatures within a 20°C to 30°C range may suggest a possible mechanism. Because van der Waals forces are not drastically affected over a 30 degree temperature range spanning physiological temperatures, physisorption is largely independent of temperature. On the other hand, because chemisorption requires heat of activation, chemisorption will vary significantly as a function of temperature. Heats of adsorption also may be measured directly or with the use of the thermodynamic law expressed as the Clausius-Clapeyron equation (Brunauer 1943). These measurements in conjunction with the adsorption isotherm analysis provide a good description of the possible mechanism for the adsorption system under examination.

Adsorption of Bacteria onto Solid Surfaces

The adsorption of bacteria onto the solid surfaces of plant cells can be divided into three temporal stages (Fletcher et al. 1980). The first stage,

simply termed adsorption, refers to events encompassing the initial approach and contact of the bacterium with the plant cell surface. The second step is one of anchoring the bacterium to a surface in a firm manner. This may be accomplished by the production of cellulose microfibrils or other polysaccharide matrices collectively known as the glycocalyx (Costerton et al. 1981). The third step, termed colonization, is a secondary phenomenon that describes the long term adsorbed state of the microorganism on the host surface. This includes continued bacterial growth and multiplication on the solid surface.

When adsorption of the microbe to living surfaces is measured, consideration should be given to the time frame under examination. For example, the adsorption characteristics after 60 min may be quite different than those measured after 2 to 4 hr, at which time microbial multiplication or metabolism of extracellular polysaccharides may greatly complicate the analysis. Therefore, if determination of the mechanism of initial adsorption is desired, incubation times should be short, i.e. well below the first doubling time, and the physiological conditions should be held constant.

The outer surface of both Gram-negative and Gram-positive microorganisms contains a complex assortment of potential attachment structures. These include outer membrane proteins, lipopolysaccharides (LPS), lipoteichoic acids, pili, and flagella. An additional structure that is less firmly associated with the bacterial surface is the capsule. Each of these components has been implicated in mediating specific attachment in any number of mammalian or marine adsorption systems (Berkeley et al. 1980, Bitton and Marshall 1980). It is of interest to note that few of these bacterial cell surface components have been studied with respect to adsorption to plant surfaces.

Exceptions include reports suggesting the involvement of LPS in both the attachment of Agrobacterium sp. to wound sites (Whatley et al. 1976) and the adsorption of Pseudomonas solanacearum to mesophyll cells (Sequeira and Graham 1977). Capsular polysaccharide-mediated adsorption of rhizobia to root hairs also has been reported (Dazzo and Hubbell 1975).

Like most colloidal particles, bacteria usually acquire a surface charge when immersed in an aqueous environment. This is due to the ionization of surface chemical groups. Due to the presence of carboxyl, amino, or phosphate groups, the charge of the bacterial surface depends upon its environment. The surface charge of the bacterium can be measured as a function of pH, providing a quantitative measure of the behavior of a particle in an electrical field (Harden and Harris 1953). Once a charge has been established on the surface, it will attract a layer of oppositely charged counter ions and generate what is known as the Stern layer (Bohn et al. 1979). Adjacent to the adsorbed Stern layer is the thick diffuse double layer. This layer contains a higher concentration of counter ions than does the rest of the aqueous phase.

The ionic characteristics of the bacterial surface, which vary as a function of ionic strength, have been shown to influence adsorption onto surfaces (Nissen 1971, Marshall 1976, Bitton and Marshall 1980, Gordon and Millero 1984, Pueppke and Benny 1984). It is now apparent that many Gram-positive and Gram-negative bacteria also exhibit some degree of hydrophobicity on their outer surface (Marshall et al. 1975, Norkrans 1981, Rosenberg et al. 1982). This characteristic appears to be an important factor in bacterial adsorption to host and inert surfaces alike (Marshall 1976, Gibbon et al. 1983, Rosenberg et al. 1983). Bacteria have been shown to adsorb to oil-water interfaces and several different inert plastic-water interfaces (Marshall et al.

1975, Marshall 1976). The contribution of hydrophobic interactions in mediating bacterial adsorption, however, remains unclear.

Adsorption of Bacterial Invaders

Plant-bacteria interactions can be separated into three groups, based primarily on the host response. In the first group no visible host response is elicited by the presence of the bacteria. This is termed a saprophytic relationship. In the second group, the interaction of a virulent bacterial strain with a susceptible host results in plant disease. This condition, which is the exception rather than the rule in nature, is termed a compatible response. Group three consists of the incompatible reaction, which is defined by the interaction of a phytopathogenic bacterium with a resistant plant. An avirulent form of the bacterium interacting with a normally susceptible host also results in an incompatible reaction.

When plant cells are disturbed by incompatible bacteria, normal host physiological functions often are altered, and affected cells rapidly collapse and die. These responses collectively are termed the hypersensitive reaction (HR). The term hypersensitivity was borrowed from medical terminology, where it describes an organism that is abnormally sensitive to a pathogenic agent (Muller 1959). The HR in plants has been known for many years (Ward 1902), and although plants exhibit the HR to a large group of fungi (Kiraly 1980) and viruses (Holmes 1929), bacterial elicitation of the HR was not documented until the 1960's (Klement and Lovrekovich 1961, 1962, Klement et al. 1964). The basic test for the HR involved injecting or vacuum infiltrating a suspension of viable bacteria (10^6 to 10^{10} cells/ml) into the intercellular spaces of a leaf. Injection is accomplished by placing the beveled end of a hypodermic syringe

containing the bacterial suspension against the leaf surface and applying pressure (Klement 1963). The intercellular spaces usually dry within 30 min, and hypersensitive flecks appear within 8 to 24 hr.

Bacterial adsorption onto the plant cell surface is associated with the initiation of the HR, and bacterial adsorption thus may trigger this plant response. The HR is only triggered by metabolically active incompatible cells (Klement and Goodman 1967). The initial step of the HR, termed the induction period, defines the time required for the irreversible activation of the HR. Plant cell-bacterial cell contact leading to bacterial adsorption is thought to be required (Cook and Stall 1977, Klement 1977, Stall and Cook 1979).

The mechanisms by which plants differentiate compatible from incompatible bacteria and initiate the HR against the latter are matters of great interest. One attractive hypothesis is that compatible bacteria multiply because they are allowed to remain free in the intercellular spaces of the leaf tissues. Incompatible bacteria, on the other hand, are thought to be adsorbed onto mesophyll cell wall surfaces and then enveloped by wall materials. These events are considered to immobilize the bacteria and to initiate the HR through an unknown series of biochemical events that culminate in bacteriostasis (Kiraly 1980, Sequeira 1980, Klement 1982). Early adsorption and immobilization events initially were described in tobacco (Nicotiana tabacum) leaves infiltrated with incompatible Pseudomonas syringae pv. pisi (Goodman and Plurad 1971, Goodman et al. 1977). During the induction period a portion of the outer cell wall separates from the mesophyll cells, apparently in response to adsorbed incompatible bacterial cells. Eventually the separated cell wall encompasses the bacteria, immobilizing them on the plant cell surface. A similar response was observed after injection of compatible and

incompatible strains of Pseudomonas solanacearum into tobacco leaves (Sequeira et al. 1977).

Data from other systems do not support the theory that adsorption and envelopment of incompatible bacteria are required to initiate the HR. For example, envelopment of the saprophyte P. putida occurs in bean, but an incompatible, HR-inducing strain of P. syringae pv. tomato is not enveloped (Sing and Schroth 1977). In addition, although compatible P. syringae pv. phaseolicola cells are not enveloped by bean, numerous bacterial cells are adsorbed onto the plant cell walls (Sing and Schroth 1977). Sequeira et al. (1977) have reported the adsorption and envelopment of non HR-inducing organisms such as Escherichia coli, Bacillus subtilis, and heat-killed incompatible Pseudomonas solanacearum by tobacco. This demonstrates that adsorption is not sufficient for induction of the HR in these systems.

Though adsorption and envelopment are of questionable significance in terms of the HR, the above data are consistent with several provisional conclusions: i) metabolically active incompatible cells are required to trigger the HR (Klement and Goodman 1967); ii) close contact and possibly bacterial adsorption onto the plant cell surface are prerequisities for induction of the HR (Klement 1977, Stall and Cook 1979); iii) because envelopment occurs in some incompatible interactions and not in others, this postulated defense mechanism does not appear to be a generalized plant response to incompatible invaders.

Adsorption of Rhizobium spp. to Root Surfaces

The ability of bacteria of the genus Rhizobium to infect the roots of legumes and produce nitrogen-fixing nodules is well documented (Fred et al. 1932). The symbiosis between legumes and members of the genus Rhizobium

generates nearly half of the 175 million metric tons of dinitrogen fixed each year by microoganisms (Hardy and Havelka 1975). The legume-Rhizobium symbiosis is controlled by both the plant (Nutman 1956, Holl and Larue 1976, Lie et al. 1981) and the bacterium (Ljunggren 1961, Maier and Brill 1976, Beringer 1980, Hobbs and Mahon 1983). This symbiosis is highly specific in that only certain Rhizobium spp. are able to nodulate any given legume species. It is this specificity which provides the basis for taxonomic groupings within the genus Rhizobium (Fred et al. 1932). Initial control of this specificity in the legume-Rhizobium sp. interaction has been postulated to occur at four to five different levels starting with regulation of growth of rhizobia in the rhizosphere and culminating with infection thread formation (Broughton 1978, Vincent 1980). Host-symbiont specificity exhibited in the legume-Rhizobium system is hypothesized to be initiated by the specific attachment of infective bacteria to the host (for reviews, see Broughton 1978, Dazzo 1980a, Solheim and Paxton 1981, Pueppke 1984a).

Substantial numbers of rhizobia adsorb, in many orientations, to root surfaces (Dazzo 1980a, 1980b, Higashi and Abe 1980, Stacey et al. 1980, Turgeon and Bauer 1982). Bal et al. (1978), however, found that only 5% of 1,798 examined root hairs contained adsorbed bacteria, and individual root hairs adsorbed only 1 to 16 bacteria. When adsorption is quantified and analyzed statistically, rhizobia are found adsorbed to both host and nonhost tissue in equivalent numbers (Chen and Phillips 1976, Bauer 1982, Law et al. 1982). In addition, both nodulating and non-nodulating strains of a given Rhizobium sp. bind to roots of the host legume (Broughton et al. 1980, Solheim 1983, Pueppke 1984b). Adsorption of four slow-growing strains of rhizobia to soybean and cowpea roots also was shown to be unrelated to the ability of the bacteria to infect and nodulate (Pueppke 1984b).

A positive correlation between bacterial adsorption and legume infection has been suggested by other workers using alternate systems. Dazzo et al. (1976) demonstrated a direct relationship between the ability of R. trifolii to infect white clover and its ability to adsorb to white clover root hairs. Specific adsorption of R. trifolii to red clover roots hairs has been correlated with the ability to infect and the presence of a symbiosis plasmid (Zurkowski 1980). Strains of R. trifolii containing the plasmid are adsorbed in much greater numbers than are strains lacking the plasmid. Several strains which have lost or reduced ability to nodulate soybean were found to have correspondingly reduced ability to adsorb to plant root tissue (Jansen van Rensburg and Strijdom 1982, Stacey et al. 1982).

The inconsistency of these experimental results may be partially explained by the widely divergent techniques and systems used to examine bacteria-root interactions. For example, bacterial adsorption to root epidermal cells may mask specific adsorption to incipient root hairs, the site of infection. Even when adsorption to root hairs is monitored directly, care should be taken to quantify adsorption only to susceptible tissues (Bhuvaneswari, 1981, Bhuvaneswari et al. 1981).

The frequently observed polar mode of attachment of the bacterium to the host's root surface was hypothesized to be of significance to infection (Sahlman and Fahraeus 1963, Dazzo 1980a, Gotz and Hess 1980, Gotz 1980). This polar attachment, however, appears to be a nonspecific phenomenon; polar binding of Rhizobium spp. occurs at oil-water and inert plastic-water interfaces (Marshall et al. 1975, Marshall 1976). The bacteria also attach in a polar fashion to roots of nonhost species such as wheat and petunia (Shimshick and Hebert 1978, Hess et al. 1982). Polar binding may simply reflect the fact that

the poles of the bacterium are more hydrophobic, favoring this type of adsorption. Charge characteristics of both the bacterium and the plant cell surface also may function in making polar attachment energetically favorable.

Fibrillar extracellular structures have been observed to encompass microcolonies of Rhizobium spp. on the surface of root cells (Reporter et al. 1975, Napoli et al. 1975). Deinema and Zevenhuizen (1971) provided evidence that some such structures are cellulose fibrils of bacterial origin. The participation of cellulose microfibrils in the initial adsorption events is probably limited, though they may serve to securely anchor the bacterium to the host in later stages of the interaction (Napoli et al. 1975, Pueppke 1984). Other substances with suspected adhesive properties also are observed in electron micrographs of the bacterium-plant interface. Marshall et al. (1975) illustrated that some type of bridging polymers bound polarly attached bacteria to the root surface. Others have seen similar structures in electron micrographs studies (Menzel et al. 1972, Dazzo and Hubbell 1975). These structures, however, also bind bacteria to inert plastic substrates. Thus, they are of bacterial origin and are induced nonspecifically (Marshall et al. 1975, Marshall 1976).

Currently there is no clearcut consensus in the literature on the relationship between host-specific adsorption and nodulation specificity. This is understandable given the fact that there exist a large number of potential control points available to both the bacterium and host from the time of bacterial root contact to nodule formation. I believe that each potential regulation site deserves careful examination, which when accomplished, will most likely suggest a concerted multi-level control with both partners participating.

Adsorption of Agrobacterium tumefaciens to Plant Surfaces

Agrobacterium tumefaciens is a Gram-negative, soil-borne, rod-shaped bacterium which causes the plant disease known as crown gall. The crown gall is a neoplastic undifferentiated growth of the plant resulting from the stable incorporation and expression of a piece of bacterial DNA (T-DNA) in the nuclear DNA of the host (Chilton et al. 1977, Gurley et al. 1979, Thomashow et al. 1980). Roughly 38% of the tested dicot families and 86% of the tested gymnosperm families, and a few monocotyledonous species, are susceptible (De Cleene and De Ley 1976). Agrobacterium tumefaciens is thought to adsorb to a specific chemical moiety in a wound site of the host's cell wall (Lippincott et al. 1977b, Rao et al. 1982). If specific adsorption does not occur, the bacterium is thought to be incapable of tumor induction. The specific adsorption hypothesis, developed by Lippincott and Lippincott (1969), provides the basis for what is known as the site-attachment hypothesis.

Most of the data in support of the site-attachment hypothesis comes from tumor formation assays employing sequential inoculations. All inoculations in these experiments are carried out by dusting pinto bean leaves with carborundum, applying 0.1 ml of bacterial suspension, and thoroughly rubbing the surface of the leaf with a glass rod (Lippincott and Heberlein 1965a, 1965b). Like many biological assays, however, the pinto bean leaf assay (PBLA) suffers from a large degree of variability from experiment to experiment. For example, the mean number of tumors per leaf after inoculation with 2.8 to 4.7 X 10^8 cells/ml varied from 4.6 to 71 to 136 in three successive experiments. This 27-fold difference in tumor number was observed, even though the inoculum densities fell within the linear range of the dose-response relationship between tumor numbers and inoculum density (Lippincott and Heberlein

1965b). Changes in plant age, greenhouse conditions, and the amount of pressure applied with the glass rod during inoculation result in a considerable difference in the number of tumors formed, and thus may account for some of the variability. Lippincott and Heberlein (1965b) suggested that an all or none assay could reduce the amount of variability associated with carborundum inoculations. Leaves could be wounded with a multiple needle holder, inoculated, and the wounds in which tumors developed scored.

The relationship between tumor numbers and initial bacterial concentration in the PBLA is linear over approximately two log units of bacterial concentration, from approximately 3×10^6 to 8×10^8 bacteria/ml. The number of tumors remains relatively constant at bacterial concentrations greater than 8×10^8 cells/ml. In this narrow range of linearity ca. 3×10^6 bacteria are required for induction of a single tumor. Additionally, the PBLA is insensitive (i.e. no tumor formation) to bacterial concentrations less than ca. 3×10^6 cells/ml (Lippincott and Heberlein 1965b). Theoretical curves for one- and two-particle events suggest that individual tumors are initiated by a single bacterium acting at a susceptible site. This would be in agreement with Hildebrand's (1942) earlier observation that a single bacterium placed on a competent plant cell can initiate tumor formation. However, this is inconsistent with the observation that tumors are not formed when inoculum densities are below ca. 3×10^6 bacteria/ml.

The extrapolation of tumor data to bacterial adsorption poses pitfalls for those attempting to understand bacterial adsorption. This difficulty has not escaped the attention of those working with the PBLA. "When more complex developments are used to measure the interaction such as disease symptom formation or the induction of host defense systems, many factors in addition to

adherence may function to determine if the specific response sought will occur. In such cases, adherence may constitute only one small part of the specificity-recognition system, or, in the extreme and even though essential, an adherence mechanism could prove so nonspecific that it would ordinarily have no role in specificity-recognition phenomena." (Lippincott and Lippincott 1980, pp. 377).

In the PBLA, virulent A. tumefaciens strains form tumors that can be easily tallied 7 days after inoculation. When pinto bean leaves were inoculated with the avirulent strain IIBNV6 prior to or in combination with, virulent strain B6, the number of tumors was reduced relative to that in controls inoculated with strain B6 alone. Preinoculation and coinoculation with strain IIBNV6 caused similar levels of inhibition. To test the possibility that such inhibition is due to simple nonspecific physical blockage by strain IIBNV6, other bacteria also were tested. Preinoculation with E. coli, Bacillus sp., and Rhizobium sp. did not reduce the number of tumors. Additionally, when the process was reversed, and the plant was inoculated with B6 prior to IIBNV6, no reduction in tumor formation was observed. These results eliminated the possibility that strain IIBNV6 nonspecifically blocked the adsorption of strain B6. In addition to IIBNV6, several virulent (heat-treated) and avirulent A. tumefaciens strains inhibit tumor formation by strain B6. Such strains are termed site-binding strains (Lippincott and Lippincott 1975). The above data provided the basis for the site-attachment hypothesis which, simply stated, suggests that there exists a single specific attachment site within a wound to which a single bacterium can attach and initiate tumor formation. Tumor inhibition, however, results from the exclusion of a single virulent cell from a specific site by a single avirulent cell. It should be emphasized that the A. tumefaciens site-

attachment hypothesis is based on tumor formation data and is not directly supported by bacterial adsorption data from the PBLA.

What appears to be a similar phenomenon, i.e. inhibition of tumor formation by inactivated cells, was noted 46 years previously (Brown 1923). No tumors formed on daisy plants puncture-inoculated with heat-killed A. tumefaciens and later inoculated in the same site with viable, virulent A. tumefaciens cells.

Enhanced tumor formation by mixtures of virulent and avirulent A. tumefaciens cells has led to some interesting, though contradictory, concepts dealing with A. tumefaciens attachment (Lippincott and Lippincott 1970, 1977a, 1978b, Lippincott et al. 1978). When pinto bean leaves were inoculated with mixtures containing virulent and avirulent cells of Agrobacterium sp., a 10 to 20-fold increase in tumor numbers was noted. The same enhancement was not observed if virulent cells were substituted for avirulent cells, i. e. if the inoculum density was increased. In these enhancement (complementation) experiments, the virulent strain is termed the donor strain, and the avirulent strain is designated the receptor strain (Lippincott and Lippincott 1970). Heat treatment of either the donor or receptor strain totally abolishes the enhancing activity. Although independent of the ratio of virulent to avirulent cells, complementation is affected by the absolute numbers of bacteria in the inoculum. For example, a 2.3:1 ratio (5×10^8 IIBNV6 cells and 2.2×10^8 B6 cells/ml) caused a 33% increase in tumor formation, but another 2.3:1 ratio (1×10^9 IIBNV6 cells and 4.4×10^8 B6 cells/ml) caused a 44% reduction in tumor formation. A ratio of 68:1 (6.4×10^9 IIBNV6 cells and 9.4×10^7 B6 cells/ml) resulted in a 63% reduction in tumor formation, yet cells in a similar ratio, 54:1 (4.5×10^9 IIBNV6 cells and 8.4×10^7 B6 cells/ml), resulted in a 40% increase in tumor formation.

Lippincott and Lippincott (1970) believe that complementation results from the separate action of a helper strain at one site and an avirulent strain at a second site. In their view, "the enhancement apparently depends on one or more substances moving between wounds occupied by virulent bacteria and wounds where IIBNV6 [receptor strain] cells are attached" (Lippincott et al. 1977a, pp. 828). Although either inhibition or enhancement can occur under conditions where ratios of virulent and avirulent cells are similar, tumor enhancement requires that the absolute number of virulent cells be relatively low, and tumor inhibition requires that the number of virulent cells be relatively high. When the concentration of the virulent strain is held constant and the concentration of the complementary avirulent receptor strain is increased, the degree of tumor enhancement increases.

The explanation for tumor enhancement is radically different from the site-attachment hypothesis proposed to explain the tumor inhibition data. Inoculation procedures are identical in both tumor enhancement and inhibition studies. When tumor inhibition is observed, competition for available attachment sites is suggested to account for the reduction in tumor formation. When tumor enhancement is observed, it is postulated that different bacteria communicate between attachment sites in separate wounds.

It would be equally plausible to hypothesize that an increased level of adsorption leads to the increased tumor formation noted in the complementation experiments, i.e. that avirulent cells facilitate the adsorption of virulent cells. This has been reported to occur when animal tissue was coinoculated with two Streptococcus mutants defective in adsorption (Larrimore et al. 1983). Although the timed inoculation data are difficult to explain, the available evidence also is consistent with the postulate that tumor inhibition is

a result of the influence of inhibitory compounds from avirulent cells on virulent cells bound to sites competent for transformation. Taken together, the results suggest that the interactions responsible for the altered tumor formation are very complex. Consequently, I believe that the evidence provides little justification to support the extrapolation to bacterial attachment in the case of tumor inhibition, or the postulation of an additional mechanism when conditions result in enhancement of tumor formation.

Proposed Molecular Components Involved in Site-attachment

The LPS on the bacterial cell surface are thought to be involved in the site-attachment process. The LPS isolated from site-binding A. tumefaciens strains by the hot phenol-water method of Westphal and Jann (1965) inhibit tumor formation. When LPS were applied to the wound sites prior to inoculation with B6, a reduction in tumor formation was observed (Whatley et al. 1976). This was not the case with LPS from non-site-binding strains. When the LPS were hydrolyzed into two components, the o-antigens and lipid A region, the o-antigens were shown to be responsible for the inhibitory activity (Whatley et al. 1976, Lippincott and Lippincott 1977).

Tumorigenicity is suppressed by introduction of plasmid pSa into A. tumefaciens (Farrand et al. 1981). More recently New et al. (1983) examined tumor inhibitory activity of strains with and without the pSa plasmid. Strains harboring pSa were no longer tumorigenic. LPS isolated from this strain also were unable to inhibit tumor formation by the virulent strain. When these strains were cured of the pSa plasmid, tumorigenicity and the ability of the isolated LPS to inhibit tumor formation were restored. Isolated LPS from strains containing and lacking pSa were compared with a variety of

techniques. Strains with and without the pSa plasmid were not qualitatively different. Carbohydrate content of the LPS was similar, and the relative amount of 2-keto-3-deoxyoctonic acid to o-antigenic polysaccharides was not significantly different (New et al. 1983).

Purity of any of these LPS fractions, however, was not established, and their compositions were not determined. The LPS isolated using the Westphal and Jann (1965) technique commonly contain 2-5% nucleic acid by weight. Detergent treatment will precipitate nucleic acids, but this procedure was not performed on the LPS used in the tumor inhibition experiments. Glucose-rich polysaccharides contaminate LPS isolated from Rhizobium sp. (Carlson et al. 1978), and LPS, regardless of the isolation procedure, always contain inorganic cations and low molecular weight basic amines, e.g. spermine, spermidine and ethanolamine (Burton and Carter 1964, Luderitz et al. 1968, Galanos and Luderitz 1975). The LPS fractions are comprised of an unpredictable mixture of different salt forms. This results in a heterogeneous LPS fraction having variable physiochemical properties that may affect biological activity (Galanos and Luderitz, 1975).

Johnson and Perry (1976) suggested five criteria for establishing LPS purity: i) elution of a single symmetrical peak at the void volume of a Sepharose 4B or 6B column, ii) the absence of ribose or deoxyribose, iii) lack of adsorption between 210 and 300 nm, iv) symmetrical elution peaks of o-antigen and core-polysaccharides from Sephadex G-50 columns, and v) a single absorbance maximum at 460 nm with the carbocyanine dye assay for LPS. The LPS material used in the tumor inhibition studies apparantly was not examined by these criteria. Attributing the observed activity to the LPS component thus, unfortunately, may be misleading.

Plant surface molecular components also were analyzed for their possible involvement in the site-attachment process. An isolated pinto bean leaf cell wall fraction inhibited tumor formation, but the plant cell membrane fraction was without activity (Lippincott et al. 1977b, Lippincott and Lippincott 1978a). Treatment of the cell wall fraction with either hot water or acid destroyed its inhibitory activity (Lippincott and Lippincott 1977, Rao et al. 1982), whereas treatment with ethylenediaminetetraacetic acid (EDTA), detergent, pectinase, or cellulase was without effect (Lippincott and Lippincott 1977). However, subsequent work from the same laboratory suggests that pectinase destroys the ability of the cell wall fraction to inhibit tumor formation (Lippincott et al. 1983).

Considerable caution, however, should be taken with results obtained using isolated crude cell wall fractions. These fractions were obtained by grinding leaves in buffer and removing the particulate wall material by low speed centrifugation. The material was then suspended in acetone, homogenized, filtered, washed three times with acetone and air dried. Similar preparations have been shown to contain 6% (w/v) protein (Slusarenko and Wood 1983). Although purity was not demonstrated for wall fractions used in the tumor inhibition assays, the authors suggest that the pectic portion of the fraction is the active component responsible for tumor inhibition. This was verified by examining several off-the-shelf commercial preparations of cell wall origin. Citrus pectin and the sodium salt of its unesterified form, polygalacturonic acid, were found to be inhibitory; cellulose and several other polysaccharides were without activity. Sodium polygalacturonate exhibited the greatest degree of tumor inhibition and was active to a concentration of 1 ng/ml. The observation that polygalacturanate is much more effective than

pectin led to the conclusion that the degree of methylation of the galacturonan residues may be responsible for the observed differences in activity (Lippincott and Lippincott 1980, Rao et al. 1983). The influence of methylation on tumor formation was examined by enzymatically removing methyl groups with a commercial preparation of pectin methylesterase. The product of this reaction had enhanced ability to inhibit tumor formation relative to pectin. On the other hand, enzymatic methylation with pectin methyltransferase resulted in reduced tumor inhibition.

In most cases cell wall fragments isolated from monocot tissue exhibited little inhibitory activity (Lippincott and Lippincott 1978a). However, upon treatment with pectin methylesterase, they became inhibitory (Lippincott and Lippincott 1978a). This suggested a possible mechanism for the well-known resistance of most monocotyledonous plants to infection by Agrobacterium spp. Thus, the apparent lack of attachment sites in monocots was hypothesized to be due to highly methylated galacturonan residues that may present a formidable barrier to tumor formation (Lippincott and Lippincott 1977, 1978a). These results led Lippincott and Lippincott (1977) to state, "The simplest direct conclusion from these data is that the cell walls of monocots, tumors and embroyonic bean tissues are sheathed with pectic substances which are sufficiently methylated that Agrobacterium does not adhere." This mechanism suggests that treatment of wound sites in monocotyledonous plants with pectinmethylesterase would expose attachment sites and render the monocot susceptible to tumor formation. Conversely, treatment of wounds in dicot tissue with pectin methyl transferase should confer some degree of resistance to Agrobacterium spp. These experiments, which would provide a direct test of the attachment site hypothesis, have not been reported.

Tumor inhibition by pectin, polygalacturonate, LPS, and avirulent site-binding A. tumefaciens strains also have been shown by others (Brown 1923, Schilperoort 1969, Glogowsky and Galsky 1978, Cooksey and Moore 1981, Pueppke and Benny 1981). Not all these reports, however, totally support the site-attachment hypothesis. For example, Glogowski and Galsky (1978) found that both E. coli and Pseudomonas fluorescens inhibited tumor formation on potato. In addition, pretreatment of Datura stramonium wounds with avirulent Agrobacterium sp. cells had no effect on tumor formation (Beaud et al. 1963). Site-binding, avirulent A. radiobactor strain K84 reduced tumor formation by 70% when applied 3 hours after inoculation with a virulent, agrocin resistant strain (El-Kady and Sule 1981).

Furthermore, others have found, in a somewhat different assay system, that only living, site-binding strains of A. tumefaciens were able to inhibit tumor formation (Schilperoort 1969, Bogers 1972, Douglas et al. 1982). These data may not necessarily conflict with previous reports, because Lippincott and Lippincott (1969) reported that the heat treated (60^{o} C, 20 min) bacterial suspensions (10^{10} to 10^{11} cells/ml) still contained 10^{4} viable cells/ml. Though present in low numbers, a population of viable cells could influence the degree of tumor formation recorded 6 to 7 days later. The number of viable cells remaining after heat treatment was not reported by Bogers (1972) or Schilperoort (1969).

It has been reported that a process similar to the proposed site attachment step in tumor formation is essential for the induction of moss gametophore formation that occurs in the presence of A. tumefaciens (Spiess et al. 1971, 1976). In the absence of a wound, gametophore induction requires physical contact with A. tumefaciens. The extent of gametophore induction is

related linearly to the concentration of bacteria and is inhibited by heat-killed virulent cells (Spiess et al. 1976). Whatley and Spiess (1977) have identified the polysaccharide portion of the LPS as the active component that mediates the attachment of A. tumefaciens to moss. Moss gametophore induction, however, is not uniquely induced by A. tumefaciens, because five different Rhizobium spp. also were active (Spiess et al. 1977a). It would prove interesting to examine the ability of a wide variety of bacteria to induce gametophore formation. Such a test would directly address the question of whether gametophore induction by A. tumefaciens is a useful model system to study early attachment events. When adsorption of non-site binding A. radiobactor stain 6467 and site-binding A. tumefaciens strain B6 onto moss protonemal filaments was monitored by scanning electron microscopy, few differences were found (Spiess et al. 1977b). On the average, the number of B6 cells bound per spore was six more than that of A. radiobactor 6467. This appears to be the strongest evidence for site-specific adsorption mediating gametophore induction.

Adsorption of Agrobacterium spp. to plant tissue culture cells

It should be emphasized that the entire preceding discussion deals with the use of tumor formation and inhibition data to define the adsorption of Agrobacterium sp. onto plant tissue. These events are separated by 7 days and may not be directly related to one another. Several workers have overcome this problem by directly measuring the attachment of A. tumefaciens onto tissue culture or mechanically separated plant cells (Matthysse et al. 1978, Smith and Hindley 1978, Ohyama et al. 1979, Douglas et al. 1982, Draper et al. 1983). In only one case, however, have workers using tissue culture cells

directly monitored bacterial adsorption under conditions where transformation is known to occur (Smith and Hindley 1978). Using cultured tobacco cells, Smith and Hindley (1978) found that both virulent C-58 and avirulent NT-1 strains bound, though C-58 was adsorbed in larger numbers. Transformation in this system was substantiated by measuring the production of nopaline in the infected tobacco cells. It was subsequently shown that wall-regenerating tobacco protoplasts can be transformed with both A. tumefaciens cells and purified Ti plasmid and then regenerated into transformed plants (Marton et al. 1979, Wullems et al. 1981, Krens et al. 1982).

Matthysse et al. (1978, 1981) examined the attachment of various A. tumefaciens strains to both carrot and tobacco tissue culture cells. Using a ratio of approximately two bacteria per carrot cell (10^4 bacterial cells/ml:5 X 10^3 plant cells/ml) Matthysse et al. (1978) observed that a larger percentage of the virulent cells bound after incubation for 2 hours than did the avirulent cells. The initial bacterial inoculum varied from 5 X 10^3 cells/ml to 1.5 X 10^4 cells/ml. If 10^4 cells/ml are assumed to be the initial inoculum, 0.5 to 0.9 cells of each of five virulent Ti plasmid-containing strains bound per plant cell. On the other hand, approximately 0.2 bacteria bound per plant cell for each of the five tested avirulent agrobacteria. Therefore, in all cases examined, more than half the plant cells would be expected to be devoid of adsorbed bacteria. Even more interesting is the fact that when the number of plant cells is increased 10-fold, there is no change in the number of bacteria adsorbed from the bacterial suspension (Matthysse and Gurlitz 1982).

Close examination of the absolute values of the adsorption data using the carrot tissue culture cells leads to some interesting observations. Plant cells adsorbed 24% of an initial inoculum containing 5 X 10^3 cells of A. tumefaciens

strain A178/ml. Thus 1.2×10^3 bacterial cells were removed from the suspension. In similar adsorption experiments with A. tumefaciens NT-1, 8-9% of the initial inoculum (1.5×10^4 cells/ml) attached to the plant surface. Thus 1.2×10^3 NT-1 cells were removed from the suspension. Therefore the actual number of virulent (A178) or avirulent (NT-1) bacterial cells bound was identical and independent of virulence.

Matthysse et al. (1978) also reported that the adsorption of site-binding strains IIBNV6 and NT-1 to tobacco was small compared to that of A. tumefaciens B6. This deviates from what would be expected if the site-attachment process as described by Lippincott and Lippincott (1969) is operative. Since prior application of IIBNV6 or NT-1 cells reduced tumor formation by strain B6, similar levels of adsorption would be postulated. The differences, however, may reflect differences in the two attachment systems. On the other hand, they may simply call into question the general applicability of the site-attachment hypothesis to systems other than pinto bean.

Matthysse et al. (1982) provided additional evidence that questions the validity of the site-attachment hypothesis for suspension culture systems. The kinetics of attachment of A. tumefaciens B6 to both glutaraldehyde-fixed carrot protoplasts and intact carrot cells from tissue culture were similar. Protoplasts were determined to be free of cell wall material by calcofluor white ST staining. Therefore, bacterial attachment to plant tissue occurred in the absence of the postulated host receptor-site, pectic wall material. However, partial synthesis of the cell wall is required for the transformation of tobacco protoplasts by intact A. tumefaciens cells (Marton et al. 1979), suggesting that A. tumefaciens attachment to carrot protoplasts is not the prelude to transformation.

Cellulose microfibril production by A. tumefaciens cells is associated with anchoring the bacterium to the surface of tissue culture plant cells and protoplasts (Matthysse et al. 1981). The ability of Agrobacterium spp. to produce microfibrils first was described by Deinema and Zevenhuizen (1971) and later suggested by Spiess et al. (1977b) to play a possible role in bacterial attachment to moss tissue. Subsequently, however, it has been shown that the ability to synthesize cellulose microfibrils is not a prerequistie for bacterial virulence or the ability to attach to plant cells (Matthysse, 1983). Since the microfibrils are not observed until approximately 60 min after inoculation, they are postulated to function in anchoring large numbers of bacterial cells, thus creating a more favorable microenvironment for the subsequent transfer of the T-DNA.

Adsorption of Agrobacterium spp. to Culture Monocot and Dicot Cells.

Comparative data on the number of bacteria adsorbed to cultured monocot and dicot cells vary from laboratory to laboratory. Matthysse and Gurlitz (1982) reported that dicot cells adsorb larger numbers of A. tumefaciens cells, but the distinction was not apparent in other studies (Ohyama et al. 1979, Draper et al. 1983). When a ratio of approximately one bacterial cell to every 100 plant cells is used, a larger percentage of the inoculum is adsorbed to carrot (Daucus carota) suspension culture cells (48%) than to corn (Zea mays) suspension culture cells (3%). However, when actual numbers are calculated from the data, 2×10^5 carrot cells bound 960 bacteria and, if it is assumed that individual plant cells bind no more than 1 bacterial cell, 99.5% of the plant cells have no bacteria adsorbed. Approximately 60 bacteria bound to the 2×10^5 corn cells, leaving 99.9% of the plant cells

without a single bacterium attached. When viewed in this manner, A. tumefaciens adsorption to monocot versus dicot tissue becomes much less distinct. This distinction was further blurred by information provided by Ohyama et al. (1979), who measured the adsorption of radioisotope-labeled A. tumefaciens B6 to a variety of monocot and dicot suspension culture cells. Corn, bromegrass (Bromus inermis), rice (Oryza sativa), and tobacco cells bound equivalent numbers of B6 cells. In each case, 1-4 mg dry wt of plant cell suspension/ml adsorbed approximately 2.3×10^6 bacteria from an initial inoculum of 3.6×10^7 cells. Soybean cells bound significantly larger numbers of B6 cells than did tobacco. It is of interest to note that intact soybean plants are resistant to infection by most Agrobacterium spp. Recently however A. tumefaciens strain A208 from Monsanto Co., St. Louis, Missouri, has been shown to induce tumor formation on soybean plants (Jill Winter, personal communication).

Adsorption of A. tumefaciens to Susceptible Host Tissue

When A. tumefaciens adsorption onto susceptible host tissue is measured directly, neither LPS nor pectic material participates in the adsorption process, as predicted by the site-attachment hypothesis (Pueppke and Benny 1984). Pueppke and Benny (1981, 1983) reported that tumor formation on potato tubers was inhibited by added galacturonans in a similar manner as seen with the PBLA. In contrast, both the degree of galacturonan methylation and the presence of LPS in the inoculum was without influence on tumor inhibition in the potato system (Pueppke and Benny 1983). A single exception was noted with the avirulent, heat-cured strain NT-1, the LPS of which was inhibitory to tumor formation. Adsorption assays consisted of measuring the level of

attachment of ^{35}S-methionine-labeled bacteria to potato tuber discs (Pueppke and Benny 1984). Four different galacturonans, which varied from 0 to 11.7% methoxy by weight, were examined for their influence on adsorption. When adsorption was measured in the presence of citrus pectin (11.7% methoxy), polygalacturonic acid (0% methoxy) and demethylpectin (2.6% methoxy), the number of adsorbed bacteria was statistically greater than that in the absence of each compound. The presence of methylpolygalacturonic acid (8.2% methoxy) in the inoculum caused no statistically significant change. Adsorption in the presence of LPS isolated from virulent and avirulent strains was not statistically different from adsorption measured in the absence of LPS. LPS from a single strain, NT-1, were enigmatic and inhibited bacterial adsorption. LPS from strain NT-1 were the only additive that inhibited both adsorption and tumor formation.

Summary of Literature Review

According to the specific site-attachment hypothesis there exists a single site within a wound to which a single virulent bacterium can attach and cause tumor formation. According to the hypothesis, tumor inhibition results from the exclusion of a single virulent cell from a specific site by a single avirulent, site-binding cell. Adsorption is hypothesized to be mediated by the binding of bacterial cell surface LPS to sparingly methylated galacturonans in wounded dicot plant cell walls. The hypothesis has several corollaries. The first is that monocotyledonous plants are resistant to A. tumefaciens because the bacteria are unable to adsorb to the plant cell surface (Lippincott and Lippincott 1977,

1978a, Rao et al. 1982). The second is that there exist two different types of avirulent A. tumefaciens cells, which can be distinguished by their abilities to bind to plant surfaces.

The site-attachment hypothesis and its corollaries have been discussed and for the most part accepted by many reviewers of A. tumefaciens tumorigenesis (Merlo 1978, Dazzo 1980a, Dazzo 1980b, Moore and Cooksey 1981, Nester and Kosuge 1981). However, the hypothesis is entirely based on indirect data from pinto bean. In addition, although adsorption has been measured directly and quantified, the experimental conditions used in these studies do not lead to transformation. Thus, there is no assurance that the measured adsorption events are relevant to T-DNA transfer.

The initial overall objective of this study was to test the site-attachment hypothesis and its corollaries. This was done (1) by measuring the adsorption of virulent, avirulent site-binding, and avirulent nonsite-binding strains to susceptible host tissue under conditions known to permit T-DNA transfer and (2) by comparing the attachment of A. tumefaciens cells to monocotyledonous and dicotyledonous plant tissues. In addition, I performed a series of experiments designed to define the mechanism of A. tumefaciens adsorption to potato tissue.

CHAPTER THREE
ADSORPTION OF AGROBACTERIUM TUMEFACIENS TO POTATO (SOLANUM TUBEROSUM) TISSUE

Introduction

Agrobacterium tumefaciens is a plant pathogen that causes a tumorigenic disease known as crown gall. This pathogen exhibits an extremely wide host range that includes 86% of the tested gymnosperm families, 38% of the tested dicot families and several monocotyledonous plants (De Cleene and De Ley 1976). Crown gall tumors are neoplastic growths that result from the transfer and stable incorporation of a piece of bacterial plasmid (T-DNA) into the plant nuclear DNA (Chilton et al. 1977, Yadau et al. 1980).

Adsorption of A. tumefaciens to the surface of the plant host is thought to be one of the earliest events leading to tumor formation (Lippincott and Lippincott 1969, 1977, 1980). Little is known of the precise adsorption mechanism, although adsorption most likely results from the interaction of both attractive and repulsive forces acting at the plant-bacterium interface. Hydrophobicity, cell surface charges, and surface polymers all may contribute to the formation of a stable host-parasite cell interface. Several authors have suggested that adsorption in this system is site-specific (Lippincott and Lippincott 1969, Lippincott et al. 1977b). Most of the supporting data are from experiments that measure the effects of various isolated plant or bacterial compounds on the ability of A. tumefaciens strain B6 to form tumors on wounded pinto bean (Phaseolus vulgaris) leaves (Whatley et al. 1976, Lippincott et al. 1977b, Rao et al. 1982). The compounds are added to the bacterial inoculum or applied prior to inoculation. After 7 days, the number of

tumors is counted. Substances that reduce the number of tumors are considered to do so because they interfere with the initial bacterial adsorption step.

The bacterial component responsible for mediating this site-specific adsorption is believed to be the o-antigen portion of the lipopolysaccharides (LPS) (Whatley et al. 1976, Whatley and Spiess 1977). Exogenously supplied LPS interfere with tumor formation and are thought to recognize some galacturonan-rich pectic portion of the wounded plant cell wall (Whatley et al. 1976, Lippincott et al. 1977a, 1980). Exogenously supplied pectic compounds also inhibit tumor formation. It has been suggested that the degree of methylation of the pectic material influences the ability of A. tumefaciens to adsorb to plant cell surfaces. Thus, the resistance of very young dicotyledonous plants and most monocotyledonous species to A. tumefaciens, is hypothesized to reflect the extent of methylation of pectic polysaccharides of the cell wall (Lippincott and Lippincott 1978a).

In a system where bacterial adsorption to potato tuber tissue is measured directly, rather than inferred from tumor experiments, most LPS and various pectic compounds have no inhibitory effect on adsorption (Pueppke and Benny 1984). However, citrus pectin actually enhanced bacterial adsorption, and LPS isolated from A. tumefaciens strain NT-1, the single exception, reduced the number of bacteria adsorbed to the potato disc surface. In this system, pectic compounds dramatically reduce tumor formation, and LPS have little or no effect (Pueppke and Benny 1981, 1983). In addition, when attachment of A. tumefaciens to monocot tissue is measured directly, the numbers of bound bacteria are comparable to those bound to potato tissue (Chapter Five this dissertation). These observations suggest that adsorption of A. tumefaciens to plant cells is less specific than previously thought.

In this investigation my objective is to provide a quantitative description of the initial binding of A. tumefaciens to potato tissue.

Materials and Methods

Adsorption Experiments

Tumorigenic A. tumefaciens strain B6, from J. A. Lippincott, Northwestern University, was maintained at 4°C on slants of the defined gluconate-mannitol medium of Bhuvaneswari et al. (1977). After incubation for 2 to 3 days A. tumefaciens cells were washed from the slants using sterile phosphate-buffered saline (PBS: 7.2 g NaCl, 2.79 g $Na_2HPO_4 \cdot 7H_2O$, 0.43 g KH_2PO_4 per liter of deionized water; final pH 7.2), centrifuged for 10 min at 7700 X G, and washed twice with PBS. The washed bacteria were resuspended in PBS and the bacterial concentration determined turbidimetrically.

The effect of initial bacterial concentration on bacterial adsorption to plant tissue was assayed by a modification of the tumorigenesis assay described previously (Pueppke and Benny 1981). Tuber discs of potato (Solanum tuberosum L. cv. Red LaSoda) (8 mm diam X 2 mm thick) were prepared aseptically. With the use of a wire basket, batches of discs (30 discs/basket) were lowered into a 100-ml beaker containing 30 ml of bacterial inoculum (10^1 to 10^9 cells/ml of PBS). The discs were agitated gently and allowed to remain undisturbed for 60 min. The basket containing the discs then was removed and immersed in two separate rinses, each containing 30 ml of sterile PBS. Each of the two rinses consisted of vigorous agitation of 4 to 5 seconds to remove any loosely attached bacterial cells. The discs were removed from the basket, blotted dry on sterile paper towels, and three randomly chosen discs were

transferred to 2 ml of PBS contained in a hand-held, ground-glass tissue homogenizer. The discs then were homogenized. Aliquots of 100 ul from each of three dilutions, previously determined to give less than 500 colonies/plate, were spread on each of five petri plates containing yest extract-mannitol agar (0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_20$, 0.1 g NaCl, 10 g mannitol, 0.4 g yeast extract per liter of deionized water). Viable colonies were counted after incubation of the plates for 36 to 48 hr at 28°C. The reported number of bacteria adsorbed at each inoculum density is the average from two to five experiments, each of which was replicated three times. Therefore, the adsorption value at each inoculum density is derived from measurements of colonies on 6 to 15 individual plates.

As a control, noninoculated discs were homogenized, and the homogenates spread on plates. Although a few colonies formed infrequently, none resembled the white, mucoid A. tumefaciens colonies. To determine the effect of homogenization on bacterial viability, a known number of bacterial cells was added to the buffer prior to homogenization. Recovery of these cells was not influenced by the homogenization step.

Desorption Experiments

Potato tuber discs were incubated in a bacterial suspension of 10^9 cells/ml of PBS for 40 min. Three discs were removed, washed as described above, and the number of attached bacteria determined. The remaining bacterial suspension, which then contained 27 tuber discs, was diluted with PBS such that the bacterial concentration was reduced by one log unit, i.e. to 10^8 cells/ml. The initial 1:1 ratio of potato discs to ml of bacterial suspension was always maintained. After 40 min another three discs were removed and the

number of adsorbed bacterial cells determined as above. Two additional desorption experiments were done in an identical manner using a stepwise reduction in bacterial density from 10^8 to 10^7 cells/ml of PBS, and 10^6 to 10^4 cells/ml of PBS.

Scanning Electron Microscopy

Potato discs for scanning electron microscopy were obtained immediately after inoculation with 2×10^7 cells of strain B6/ml of PBS. Discs were fixed overnight at 4°C in 4% glutaraldehyde and rinsed with 0.05 M cacodylate buffer at pH 7.2. Samples then were fixed in OsO_4 for 2 hr at room temperature, washed with cacodylate buffer, and dehydrated through an ascending series of ethanol solutions. Samples were critical-point dried by substitution of ethanol with liquid CO_2, sputter coated with gold, and viewed with a Hitachi 450 Scanning electron microscope at 20 KV.

Results

Bacterial Adsorption and Desorption

Depending on initial bacterial concentration, 0.23×10^1 to 1.2×10^7 bacteria adsorbed per disc (Table 1). The percentage of bacteria adsorbed from suspensions containing from 10^1 to 10^4 cells/ml of PBS varied from 23 to 5. Approximately 1% of the inoculum was adsorbed at bacterial concentrations greater than 10^5 cells/ml of PBS (Table 1). Saturation was never observed in these experiments. Adsorption studies also were carried out using potato discs

Table 1. Adsorption of Agrobacterium tumefaciens to Potato Tissue.

Initial Bacterial Concentration (cells/ml)	No. of Bacteria Adsorbed/Disc	
	Measured[a] (%)	Predicted[b]
10^1	0.23×10^1 (23.0)	0.13×10^1
5×10^1	0.55×10^1 (11.0)	0.57×10^1
10^2	0.85×10^1 (9.0)	1.00×10^1
5×10^2	7.50×10^1 (15.0)	3.60×10^1
10^3	7.80×10^1 (8.0)	6.70×10^1
5×10^3	3.30×10^2 (7.0)	2.60×10^2
10^4	5.10×10^2 (5.0)	4.70×10^2
10^5	1.60×10^3 (1.6)	3.20×10^3
10^6	1.10×10^4 (1.1)	2.00×10^4
10^7	1.30×10^5 (1.3)	1.40×10^5
5×10^7	5.80×10^5 (1.2)	5.30×10^5
10^8	1.30×10^6 (1.3)	9.40×10^5
5×10^8	6.30×10^6 (1.3)	3.50×10^6
10^9	1.20×10^7 (1.2)	6.20×10^6

[a] The data are from four separate experiments. Three discs were homogenized at each sampling time, and each homoginate was spread on five plates.
[b] Predictions are based on the Freundlich adsorption isotherm, $C_b = 0.251 C_s^{0.822}$.

with a 43% smaller surface area than those used in most experiments. When these smaller discs were exposed to bacterial densities up to 10^9 cells/ml of PBS, saturation of the available adsorption sites was not reached.

When bacterial suspensions in equilibrium with potato discs were diluted from 10^9 to 10^8 cells/ml, there was coincident reduction in the number of bacterial cells adsorbed to the discs (Table 2). The number of bacteria adsorbed per disc also decreased after the 10-fold dilution from 10^8 to 10^7 cells/ml. A 100-fold dilution from 10^6 to 10^4 cells/ml similarly resulted in a reduction in the number of cells bound per disc. These data indicate that binding is reversible.

Theoretical Considerations

Before experimental data can be analyzed with respect to adsorption isotherms, several preconditions about the adsorption system under analysis must be met (Brunauer 1943, Saunders 1971, Tompkins 1978). First, the total number of adsorption sites should be constant under all experimental conditions. This condition is met because discs of the same size were used in each experiment. Second, adsorption must be restricted to a monolayer. This was confirmed by examination of inoculated potato tuber surfaces by scanning electron microscopy (Fig. 1). The existence of monolayer adsorption eliminates the possible use of the Brunauer-Emmett-Teller adsorption isotherm, which describes multilayer adsorption phenomena (Brunauer 1943). Third, every adsorption site should be equivalent, and the ability of the bacteria to adsorb must be independent of whether or not adjacent sites are occupied. These conditions also appear to be fulfilled. Potato tuber tissue is relatively uniform,

Table 2. Desorption of Agrobacterium tumefaciens Cells from Potato Tuber Discs.[a]

Initial No. Bacteria		Final No. Bacteria	
Per ml of Inoculum	Adsorbed/Disc	Per ml of Inoculum	Adsorbed/Disc
10^9	$2.8 \pm 0.3 \times 10^7$	10^8	$5.9 \pm 1.3 \times 10^6$
10^8	$2.0 \pm 0.5 \times 10^6$	10^7	$9.7 \pm 1.3 \times 10^5$
10^6	$1.4 \pm 0.2 \times 10^4$	10^2	$6.0 \pm 0.3 \times 10^3$

[a] The data are from separate experiments. Three discs were homogenized at each sample time, and each homogenate was spread on plates.

[b] Standard Deviation.

Fig. 1. Scanning electron micrograph of Agrobacterium tumefaciens strain B6 on potato tuber tissue. The initial inoculum density was 2×10^9 bacteria/ml and the disc was fixed immediately after inoculation. The field of view presented here is 870 um^2 and contains 12 rod-shaped bacteria. X 5200.

and it would be expected that adsorption sites would be reasonably equivalent. Moreover, bacterial adsorption to the discs did not reach saturation, even when small discs were exposed to very high bacterial densities. This makes it unlikely that the plant cell surfaces were packed with bacteria and that occupation of adjacent sites was of importance. These observations provide evidence for the equivalency of adsorption sites. Fourth, the rate of adsorption to unoccupied sites must equal the rate of desorption from occupied sites at equilibrium, i.e. equilibrium must be reversible. This is illustrated in Table 2.

The binding of A. tumefaciens strain B6 to potato tuber tissue can be described best in terms of the Freundlich adsorption isotherm (Brunauer 1943, Tompkins, 1978), which is expressed as

$$C_b = k_1 \times Cs^{1/k_2}$$

where C_b = the number of bacterial bound per disc,

C_s = the concentration of the bacteria in the suspension at equilibrium,

k_1 and k_2 are both constants.

When a linear regression is performed on the plot of log C_s vs log C_b, a correlation coefficient of r = 0.987 is obtained for the entire range of inoculum densities studied. Demonstration of the linear relationship between log C_s vs log C_b is in accordance with the criteria set forth for the Freudlich isotherm (Brunanuer 1943, Tompkins 1978).

When the data are fitted to three other isotherms (Langmuir, Temkin, or Henry's law), the curves fall outside acceptable levels of confidence. The Temkin isotherm requires the relationship between log C_s and C_b to be linear. My data yield a correlation coefficient of r = 0.60. The Langmuir isotherm requires the plot of C_s/C_b vs C_s to be linear. My data yield a correlation

coefficient of r = 0.39. Henry's law defines a linear relationship over the entire isotherm. The potato-A. tumefaciens adsorption isotherm is nonlinear in the range of 10^1 to 10^5 cells/ml of PBS (Table 1). Therefore, none of these three alternative isotherms adequately describes the experimental data for the entire inoculum range studied.

When theoretical isotherm plots of bound versus free bacteria for each of the isotherms in Fig. 2 are compared to the experimental data presented in Table 1, the empirical correspondence of the data to the Freundlich isotherm plot is very good. Similar to Fig. 2a, the experimental data show a rapid increase in bacterial adsorption per unit increase in initial inoculum density from 10^1 to 10^4. At higher initial bacterial densities, in the range of 10^5 to 10^9 cells/ml of PBS, the numbers of bacteria adsorbed are linearly related to the initial inoculum density. At all high bacterial densities, approximately 1.2% of the bacteria adsorb to the potato discs. This simple linear part of the adsorption isotherm is described best by Henry's law: $C_b = k_1 \times C_s$ (Fig. 2c). Saturation as predicted by the Langmuir isotherm (Fig. 2a) is not reached. Linear regression analysis of log C_s vs log C_b was used to determine the slope and y-intercept. These values then were used to determine values of the constants k_1 and k_2 of the Freundlich isotherm over the entire range of bacterial concentrations (10^1 to 10^9 cells/ml of PBS):

$$C_b = k_1 \times C_s^{\,1/k2}$$

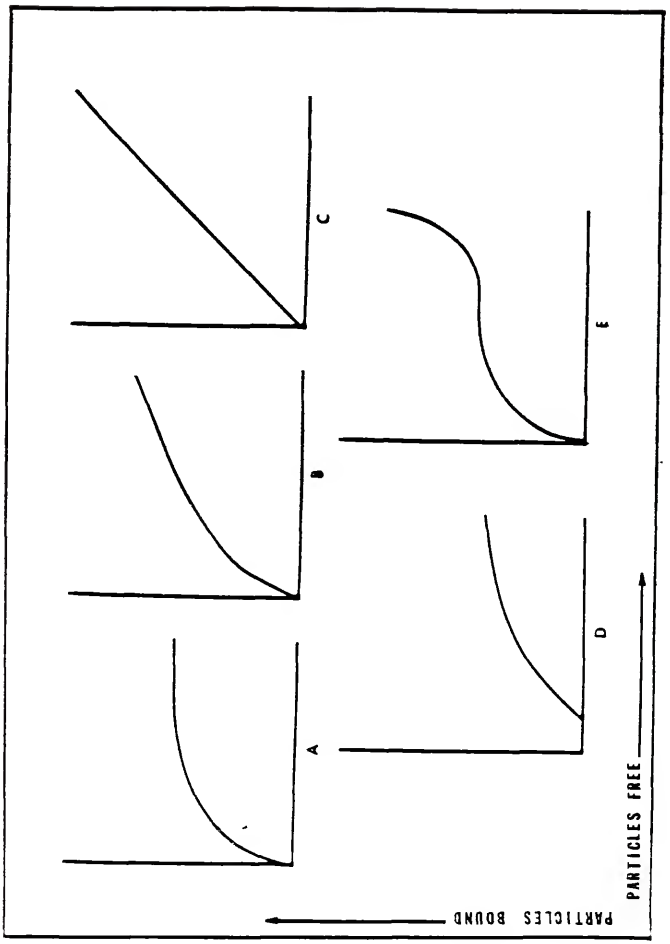
When $\log C_b = \log k_1 + 1/k_2 \log C_s$

Slope $1/k_2 = 0.822$

y-intercept $\log k_1 = -0.601$

$k_1 = 0.251$

Fig. 2. Empirical plots of five adsorption isotherms.
A) Langmuir B) Freundlich C) Henry's law D) Temkin E) Brunauer Emmett Teller


C
E
B
D
A
PARTICLES FREE
PARTICLES BOUND

Thus the Freundlich adsorption isotherm for the adsorption of A. tumefaciens to potato tuber discs is

$$C_b = 0.251 \times C_s^{1/1.217} = 0.251 \times C_s^{0.822}. \quad (a)$$

Using equation (a) a predicted curve of log C_s vs log C_b was plotted, and the experimental data points superimposed (Fig. 3). The predicted number of adsorbed bacteria per disc at each inoculum density is listed in Table 1. A curve fit to the experimental data points using the Statistical Analysis System (SAS) nonlinear curve fitting procedures (NLIN) generated

$$C_b = (0.098) \times C_s^{0.897}.$$

for parameters $k_1 = 0.098$ and $1/k_2 = 0.897$, asymptotic 95% confidence intervals are -0.062 to 0.257 and 0.984 to 0.823 respectively. Clearly, the parameters k_1 and $1/k_2$ generated from the log C_s vs log C_b plot fall within these confidence intervals.

Discussion

The binding of cells of A. tumefaciens strain B6 to potato tuber tissue is a reversible equilibrium process. This binding can be described accurately in terms of the Freundlich adsorption isotherm, which is a non-ideal variant of the Langmuir adsorption isotherm (Brunauer 1943). Over the wide range of initial bacterial concentrations examined, non-saturation of available binding sites is in accordance with the Freundlich isotherm.

Adsorption of cells of Pseudomonas lachrymans onto leaf surfaces is nonspecific and nonsaturating over inoculum densities of 10^4 to 10^{10} cells/leaf (Haas and Rotem 1976). Leben and Whitmoyer (1979) also observed that the number of adsorbed P. lachrymans cells is related linearly to the number of cells applied. Fletcher (1977) suggested that the initial adsorption of marine

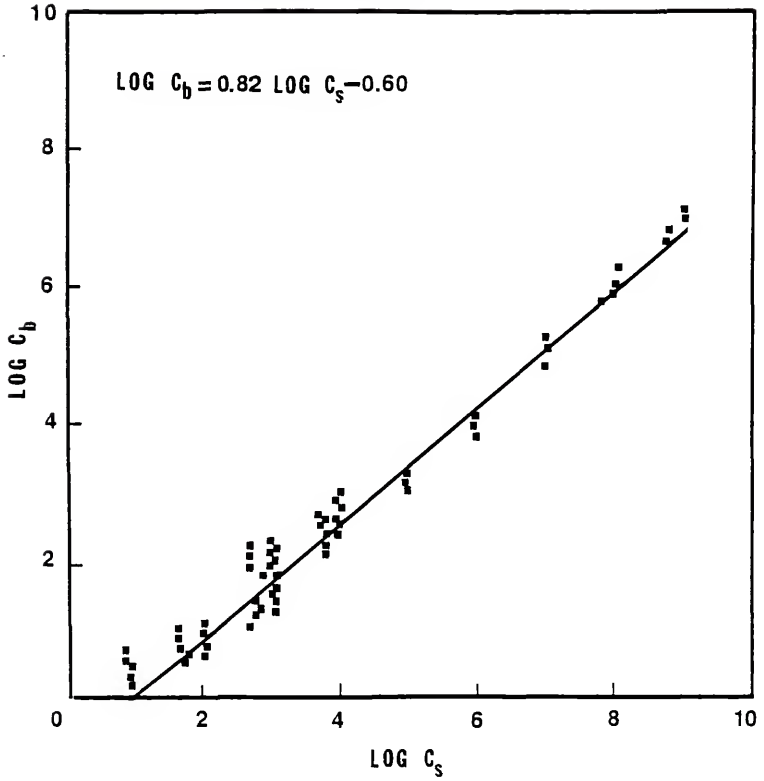


Fig. 3. The Freudlich adsorption isotherm for Agrobacterium tumefaciens strain B6 on potato tuber tissues. The line is the predicted adsorption curve on log C_s vs log C_b generated from equation (a). The experimental data points are from Table 3.

pseudomonads to nonbiological surfaces is driven by nonbiological, physiochemical adsorption forces as described by a modified Langmuir-like adsorption isotherm. In addition, the adsorption of Rhizobium spp. to cereal roots was described by the Langmuir isotherm and hypothesized to be influenced by nonspecific, as opposed to specific binding events (Shimshick and Hebert 1978, 1979). My data similarly point to the importance of nonspecific forces in the adsorption of A. tumefaciens to potato tissue.

The experimental data presented here do not lend support to the specific site attachment hypothesis advanced by Lippincott and Lippincott (1969, 1977, 1980). According to these authors, there exist in wounded pinto bean leaves a small, finite number of specific binding sites for tumorigenic A. tumefaciens cells. Heat-treated, tumorigenic A. tumefaciens cells and cells of certain nontumorigenic Agrobacterium sp. strains are hypothesized to be capable of occupying these sites and excluding pathogenic cells from them. Coinoculation studies using unlabeled bacteria in competition with radioisotope-labeled bacteria indicated that cells are not competing for a limited number of specific sites on the plant surface (Pueppke, unpublished observations). The data also indicate that cut surfaces of potato tuber discs contain an extremely large number of nonspecific adsorption sites for A. tumefaciens. Additionally, if a specific site-attachment phenomenon is operative, one would expect bacterial adsorption to be saturable as described by the Langmuir isotherm. This clearly is not the case with adsorption of A. tumefaciens to potato tuber tissue.

There are three possible explanations for the discrepancies between the data presented here and those of Lippincott and associates. The first is simply that the adsorption of A. tumefaciens to potato discs is much different than that to pinto bean leaves. Although this is possible, A. tumefaciens infects and

produces tumors on both plants, and similar infection mechanisms are to be expected. The second explanation is that Lippincott and associates may have been misled by their extrapolation of tumor data to the adsorption phenomenon. Many events intervene between initial bacterial adsorption, which occurs rapidly after inoculation, and the appearance of tumors, which occurs 7 to 14 days after inoculation. It is to be expected that some treatments would influence tumor development without having an effect on adsorption. Pueppke and Benny (1984) directly monitored adsorption of A. tumefaciens to potato tissue in the presence of either LPS or various pectic components. Pectic components reduced the numbers of tumors formed on potato and all but one LPS preparation was without effect (Pueppke and Benny 1983). In nearly every case, however, these substances did not reduce bacterial adsorption, suggesting that pectic materials influence some step subsequent to adsorption. The existence of two types of binding sites is a third possible explanation. A small number of highly specific irreversible adsorption sites that lead to tumor formation may coexist with a large number of nonspecific, reversible adsorption sites.

The dose-response relationship between inoculum concentration and tumor number is linear at bacterial concentrations from 10^5 to 10^8 bacteria/ml but the response saturates at higher bacterial concentrations (Pueppke and Benny 1981). In contrast, the relationship between inoculum concentration and adsorption is linear at bacterial concentrations from 10^5 to 10^9 bacteria/ml. Thus, there is a lower threshold below which tumors do not form and an upper threshold above which the tumor response does not keep pace with increases in adsorption. These differences are not surprising, given the complex nature of tumorigenesis relative to that of adsorption and the fact that tumors coalesce

and become unresolvable at bacterial concentrations above 10^8 bacteria/ml (Pueppke and Benny 1981). The effect of inoculum concentration on the adsorption of rhizobia to soybean roots (Pueppke 1984b) and the subsequent nodulation response (Perkins 1925) appears to be similar.

It should be noted that adsorption isotherms are only empirical expressions that reasonably approximate the behavior of experimental binding systems over restricted concentration ranges. Although the Freundlich isotherm was derived for nonbiological systems, the compliance of my data with the necessary assumptions and principles of this model underscores the importance of nonspecific physical forces in A. tumefaciens adsorption. Although it is conceivable that the good fit of the experimental data to the Freundlich adsorption isotherm is fortuitous, the data justify further examination of bacterial adsorption in terms of nonspecific physical forces. Adherence to a particular adsorption isotherm does not predicate a particular type of binding for the system. Adsorption described by the Freundlich adsorption isotherm, for example, does not imply that a physisorption or a chemisorption process is operative. Rather, the isotherm should permit useful inferences to be drawn and parameters to be set for further testing of bacterial adsorption to plant surfaces.

CHAPTER FOUR

THERMODYNAMICS OF *AGROBACTERIUM TUMEFACIENS* ADSORPTION TO PLANT SURFACES

Introduction

The adsorption of bacteria to plant cell surfaces is acknowledged to play an important role in the ecology of prokaryote-plant interactions (Dazzo 1980a, Pueppke 1984a). Before legume nodulation can occur, *Rhizobium* spp. must adsorb to the root hair surface to initiate the nodulation process (Schmidt 1979, Bhuvaneswari 1981). Bacteria also adsorb quite tenaciously to leaf surfaces (Haas and Rotem 1976, Leben and Whitmoyer 1979). The differential adsorption of *Xanthomonas* spp. or *Pseudomonas* spp. to mesophyll cell surfaces may determine whether a disease or hypersensitive response will occur (Klement 1982).

Additionally, tumor formation studies imply that site-specific adsorption of *A. tumefaciens* to plant wounds is required for plant cell transformation (Lippincott and Lippincott 1969). In view of the fact that a piece of the bacterial T-DNA is mobilized and incorporated into the plant nuclear genome, adsorption to the plant cell surface appears to be a logical precondition for transformation. It has been shown that *A. tumefaciens* adsorbs to potato tissue in very large numbers (Chapter Three this dissertation, Pueppke and Benny 1984) and that adsorption can be described in terms of the Freundlich adsorption isotherm (Chapter Three this dissertation). However, the basic mechanism governing the adsorption of *A. tumefaciens* is poorly understood.

The objective here was to describe the mechanism of A. tumefaciens adsorption in terms of physi- or chemisorption.

Materials and Methods

Maintenance of Bacteria and Preparation of Inocula

Agrobacterium tumefaciens strain B6 was maintained at 6°C on glutamate-mannitol agar slants. For all adsorption experiments bacteria were washed from 4- to 5-day-old slants with phosphate-buffered saline (PBS), centrifuged at 8,000 X G, and resuspended in PBS to the desired bacterial concentration. Bacterial concentration was determined turbidimetrically.

Effect of Temperature on Adsorption

Adsorption experiments at elevated temperatures were done in a 38°C water bath. Before the adsorption experiment, the bacterial suspension and PBS solutions to be used for rinsing were equilibrated to 38°C in the water bath for 25 min. Potato discs were placed in a covered plastic petri dish, which was floated in the 38°C water bath for approximately 3 min to equilibrate to 38°C. The 6°C adsorption experiments were carried out in a refrigerator, where all washes, and bacterial suspensions were equilibrated to 6°C for 25 min prior to the adsorption assay. Potato discs were placed in a covered plastic petri dish and stored at 6°C for 3 min prior to the adsorption assay. The room temperature experiments were done at 28°C. The adsorption isotherm experiments were repeated three times at each of the three temperatures.

The entire adsorption assay used here has been described in detail elsewhere (Chapter Three this dissertation). After a 60 min adsorption period, discs were washed three times in consecutive 30-ml portions of PBS, ground in PBS, serially diluted and plated onto yeast extract mannitol (YEM) agar plates. Viable colonies were counted after incubation for 36 to 48 hr at 28°C. At each inoculum density the value reported is the average of three replications. The number of viable colonies in four plates at three different dilutions was tallied to determine the number of bacterial cells adsorbed per potato disc.

Kinetic Binding Experiments

These experiments employed the adsorption assay described above. However, the binding periods were interrupted at predetermined intervals (0.5, 1, 5, 10, 30, and 60 min) and the number of adsorbed bacteria determined. Baskets containing the discs immersed in the bacterial suspension (2×10^7 bacteria/ml) were removed after the prescribed binding period and rinsed as described above. The number of bacteria bound per disc then was determined as above.

Desorption Experiments

After incubation of the discs with 2×10^7 bacteria/ml for 30 min the discs were removed and quickly washed in sterile PBS for 2 to 3 sec. Each disc then was placed in a 1.5-cm X 12.5-cm test tube containing 2 ml of sterile PBS at 6°C. Aliquots of 100 ul were withdrawn at specific intervals over a 10.5-hr period. At each sample time the disc was gently swirled before the sample was

withdrawn. These aliquots were either plated directly onto YEM agar or serially diluted prior to plating. The number of bacteria adsorbed per disc prior to and after the desorption period was determined as described previously (Chapter Three this dissertation). The experiment was conducted twice with three replicates taken at each sample time.

Sequential Adsorption

The standard adsorption assay using A. tumefaciens strain B6 was used with the following modifications. A bacterial suspension containing 2×10^7 bacteria/ml was used to inoculate one set of discs at the ratio of one disc/ml of bacterial suspension. After removal of the discs, another set of discs was placed into the inoculum. After removal of the second set of discs, the bacterial suspension was used to inoculate a third set of freshly prepared potato tuber discs. Individual discs from all three sets were homogenized, and the number of bacteria adsorbed per disc was determined. The adsorption period was 30 min, and the temperature was 28°C. The sequential adsorption experiment was repeated twice with four replications in each repitition.

Zeta Potential Measurements

Bacteria were washed three times with sterile, deionized water, centrifuged at 8000 X G for 12 min and resuspended in sterile, deionized water. The bacteria were adjusted to 5×10^7 cells/ml of sterile deionized water, and then titrated to the desired pH with KOH or HNO_3 just prior to measurement of the zeta potential. Each suspension was injected into the electrode chamber of a Laser Zee Meter Model 502, and the focal plane above

the inside bottom of the electrode cell adjusted to 873 um. All zeta potential measurements were taken in this focal plane, the upper stationary phase of the cell. The zeta potential of the bacteria was measured using an electrical field of 75 volts. Two to three samples were measured at each pH, and three to five measurements of each sample were recorded. After all measurements were made, the sample was decanted and the pH remeasured. Deviation of greater than 0.1 pH unit was not observed. Due to the relative transparency of the bacteria to the laser beam across the cell, the bacteria were visualized with substage tungsten illumination. A video camera projected the image onto a video monitor where bacterial mobility in the electrical field was observed.

Results

The adsorption of A. tumefaciens was measured at 6°C, 28°C, and 38°C. The adsorption isotherms obtained at each of these three temperatures, using bacterial concentrations spanning three orders of magnitude, are illustrated in Fig. 4. Least squares regression analysis using the General Linear Model of the Statistical Analysis System was used to compare slopes and y-intercepts of the isotherms. Log-transformed data of both the initial inoculum and number adsorbed were used in the regression analysis, and temperature was considered to be a class variable. Temperature did not significantly alter the y-intercepts of the three isotherms at $P = 0.05$. The slopes, however, were significantly different at $P = 0.05$, but not at $P = 0.045$. When the same analysis was carried

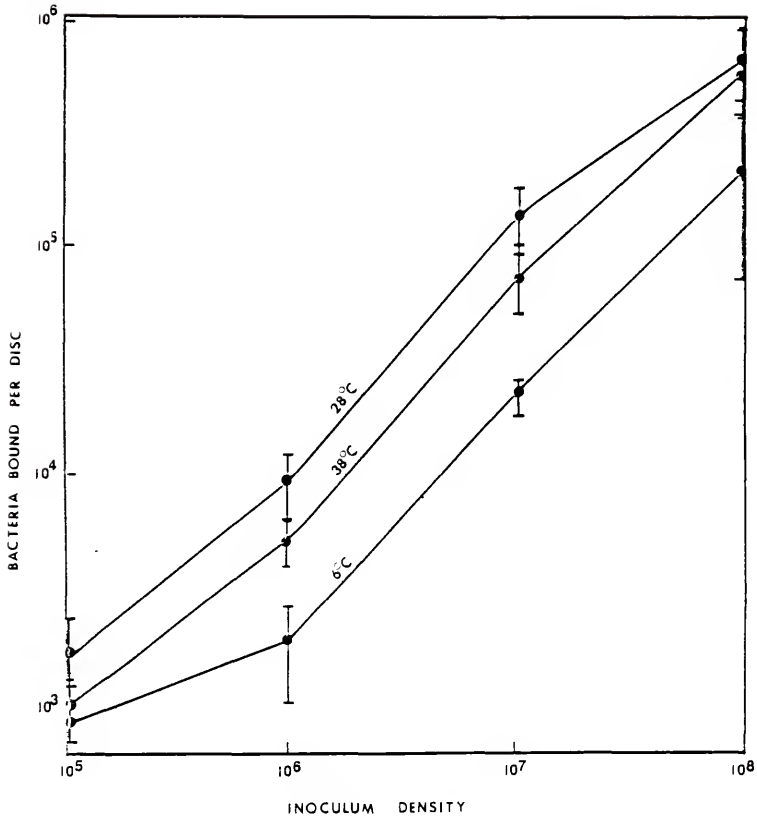


Fig. 4. Isotherms for the adsorption of Agrobacterium tumefaciens cells to potato tissue at three different temperatures. Each point is the mean of measurements taken in three separate experiments with three replications in each experiment. Bars represent the standard errors of the means.

out using the 28°C and 38°C isotherms, neither the slopes nor the y-intercepts were significantly different (Zarr 1974). Comparison of these isotherms using simple linear regression is valid, because at these bacterial concentrations the adsorption isotherm is described by a linear relationship, Henry's law: $C_b = kC_s$ (Brunauer 1943, Chapter Three this dissertation).

Isosteric heats of adsorption were determined using the Clausius-Clapeyron equation expressed as

$$\Delta H/R \times d(1/T) = d\ln C_s$$

where ΔH = change in heat content of the system;

R = the gas constant, 0.082 l-atm/mole K;

T = degrees Kelvin;

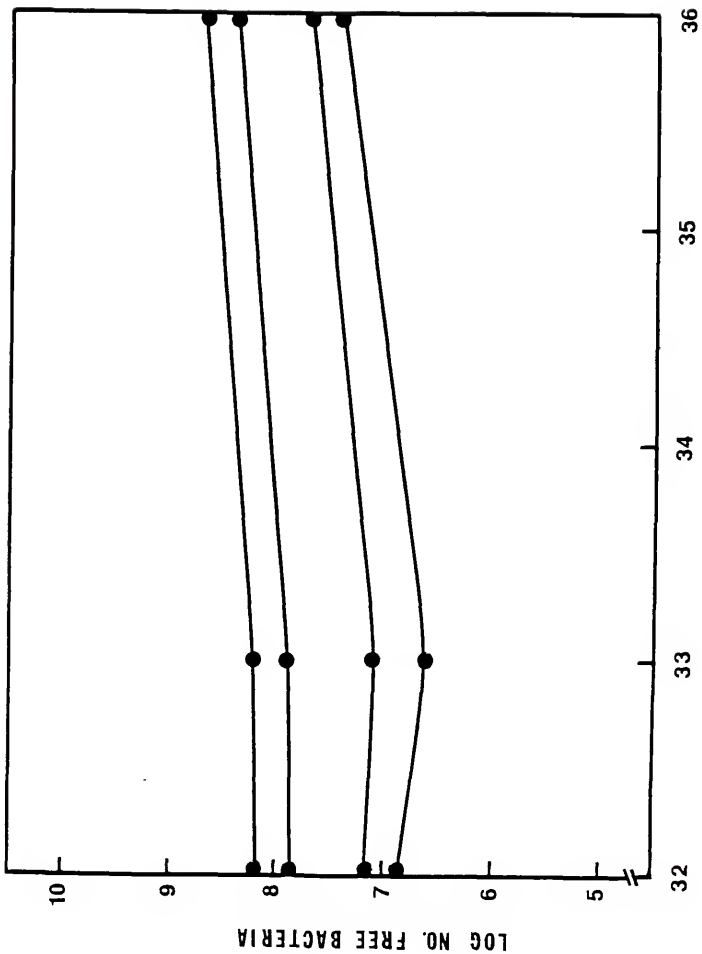
C_s = [free bacteria] eq, the concentration of unbound bacteria at equilibrium.

In the form of a straight line the expression becomes

$$\ln C_s = \Delta H/R \times 1/T.$$

The concentration of free bacteria at equilibrium at a given level of bacterial adsorption for each of the three temperatures examined is plotted against 1/T in Fig. 5. In plots of log C_s against 1/T, at a constant level of bacterial adsorption, H/R is the slope of a line that allows the heat of adsorption to be derived. The correlation coefficient for each of the four lines plotted in Fig. 5 is greater than r = 0.91. Analysis of variance shows the slopes of this family of lines not to be different from one another and not to be statistically different from zero. This indicates that the heat of adsorption is exceedingly small.

Fig. 5. Transformation of the data in Fig. 4 using the Clausius-Clapeyron equation. T is the temperature in degrees Kelvin.


LOG NO. FREE BACTERIA
10
9
8
7
6
5
32
33
34
35
36
$\frac{1}{T} \times 10^4$, K

Kinetics of Adsorption

The number of bacteria adsorbed per disc was measured as a function of the adsorption period, which is defined as the period of time during which the potato discs are immersed in the bacterial suspension. More than 2.8×10^4 cells bound per disc after incubation for 30 sec (Table 3). The number of bacteria adsorbed to the surface continued to increase with time. However, there is no significant difference between the number of cells bound during a 30 min or a 60 min adsorption period.

Desorption Experiments

The adsorption of A. tumefaciens to potato tuber tissue can be described by the Freundlich adsorption isotherm (Chapter Three this dissertation). Such a dynamic equilibrium model implies that the adsorbed bacteria must desorb and undergo a reversible binding. Bacterial desorption was measured over a period of 10 hr at 6°C to reduce interference due to bacterial multiplication (Fig. 6). Bacterial desorption is not linearly related to time. After 4 to 5 hr the desorption curve flattens as equilibrium is approached, i.e. the rates of desorption and adsorption are equal (Fig. 6). By 10.5 hr approximately 31% of the bound bacteria (5.8×10^4 of 1.9×10^5 bacteria) had desorbed from each disc.

Sequential Adsorption

A single bacterial suspension was used to sequentially inoculate three groups of freshly cut potato tuber discs. The mean number of bacteria

Table 3. Time Dependence of the Adsorption of Agrobacterium tumefaciens B6 to Potato Tuber Tissue.

Adsorption Period[a] (Min.)	Number of Bacteria ($\times 10^5$) Adsorbed per Disc[b]
0.5	0.29 ± 0.06 a
1.0	0.46 ± 0.09 b
10.0	1.44 ± 0.73 c
30.0	2.60 ± 0.28 d
60.0	2.50 ± 0.40 d

[a] All adsorption experiments were carried out with an initial inoculum of 2×10^7 cells/ml.

[b] Numbers followed by the same letter are not significantly different ($p = 0.05$) according to Student's t-test.

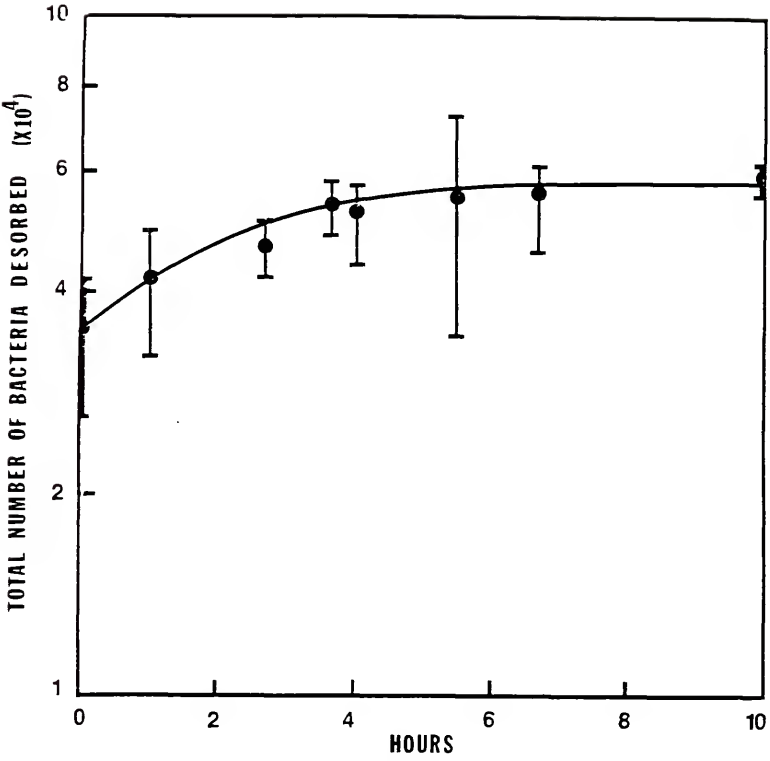


Fig. 6. Desorption of Agrobacterium tumefaciens from potato tuber disc surfaces. The log of the total number of bacterial cells desorbed from the surface is plotted against the time in hours. Bars represent ± 1 standard deviation from the mean.

adsorbed per disc (± standard deviation) for groups one, two, and three was 9.8 ± 4.1 X 10^4, 1.1 ± 0.43 X 10^5, and 1.5 ± 0.66 X 10^5, respectively. There were no significant differences between any of the three groups at the 1% level, when analyzed using Student's t-test (Fig. 7).

Zeta Potential Measurements

Zeta potential measurements of bacteria suspended in deionized water indicate an isoelectric point (pI) of approximately 2.9 (Fig. 8). Above pH 2.9 the bacterial cell surface was strongly negative. A zeta potential of greater than -30 mV was observed for values above pH 5.0. Below pH 2.9 the bacterial cell surface was positively charged. However, the magnitude of the positive charge was not as great as that of the negative charge at an equivalent distance above the pI.

Discussion

Although Agrobacterium tumefaciens adsorption can be described by the Freundlich adsorption isotherm (Chapter Three this dissertation), this does not imply an adsorption mechanism for the system. The basic effort in this communication was to define the mechanism of adsorption of A. tumefaciens to potato tuber tissue by distinguishing between physisorption and chemisorption. I have attempted to do this by comparing and contrasting my experimental results with the salient features of each of these two basic adsorption types. Bacterial adsorption to potato discs occurs very rapidly and in large numbers at

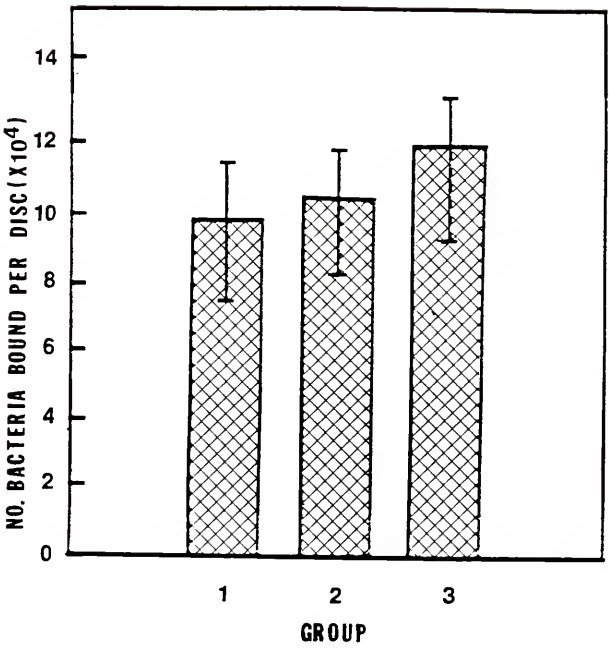


Fig. 7. Sequential adsorption assay. Three discs were sampled in each sequential group, and the experiment was done twice. Bars represent $\pm$ 1 standard deviation from the mean.

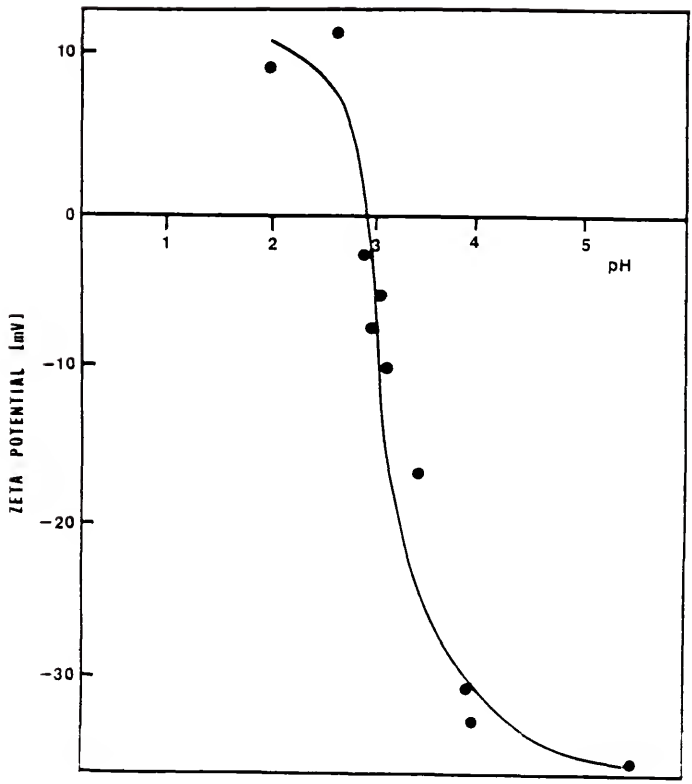


Fig. 8. Zeta potential measurements. Each point represents the mean of four measurements taken for each of the three samples.

bound per disc. Unless the heat of activation for bacterial adsorption is extremely small, these data suggest that physisorption is operative.

Most chemisorbed particles are irreversibly bound and exceedingly difficult to remove (Brunauer 1943). Adsorption of A. tumefaciens, however, is reversible and approaches an equilibrium. Adsorption assays done at 6°C, 28°, and 38°C indicate that temperature does not have a significant effect on the number of bacteria bound. The heat of adsorption as determined with the Clausius-Clapeyron equation is nearly zero, well below the 10-20 kcal value arbitrarily established as the point below which physisorption most likely predominates (Brunauer 1943, Saunders 1971). These data all indicate that the binding of A. tumefaciens can be described in terms of physisorption.

If the adsorption process is to be favored, the free energy of the system will decrease as particles are adsorbed onto the surface. This concept is related by the expression;

$$\Delta F^{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL}$$

where ΔF^{adh} is the free energy of adsorption, γ_{BS} is the bacterium-substratum interfacial tension, γ_{BL} is the bacterium-liquid interfacial tension, and γ_{SL} is the substratum-liquid interfacial tension. This simple thermodynamic model for the adsorption of small particles has been shown to describe the qualitative features of bacterial adsorption (Absolom et al. 1983). This further illustrates that bacterial adsorption can be viewed in thermodynamic terms and related to colloidal adsorption systems.

Statistical analysis of data taken over a 32°C temperature range suggests little or no effect of temperature on the actual number of bacteria bound. Ohyama et al. (1979) also found little effect of temperature (24° to 35°C) on the number of A. tumefaciens cells bound to plant suspension culture cells.

Similarly, agglutination of asparagus cells by A. tumefaciens appeared to be independent of temperature (Draper et al. 1983). This would suggest that an ionic interaction is one of the forces involved in the adsorption process, a characteristic of physisorption.

Since physisorption appears to be operative in this system, characterization of the ionic nature of the bacterial surface indicate a pI of 2.9 in distilled water. Harden and Harris (1953) gathered data on the isoelectric point of 21 species of Gram-negative bacteria and found pI values ranged from 2.07 to 3.65. At pH values below the pI, the A. tumefaciens surface exhibits a small net positive charge. This may be due to the fact that few ionizable amino groups are present on the bacterial surface. At pH values above the pI, a large number of carboxyl and/or phosphate groups may be ionized and account for the large net negative charge. These data would then suggest that a very high ratio of carboxyl or phosphate to amino groups is present on the bacterial surface.

Since the pH of the sap in potato tuber wounds is 5 (unpublished results), which is above the isoelectric point of A. tumefaciens, the bacterial cell is most likely negatively charged within the wound site. However, there are a large number of components within the wound which, when adsorbed onto the bacterial surface, may alter the bacterial charge characteristics. The possible role of these surface charges in adsorption remains to be defined.

At inoculum densities that fall within the linear range of the A. tumefaciens adsorption isotherm, 10^5 to 10^9 bacteria/ml, a relatively constant 1.2% of the inoculum is adsorbed from the inoculum (Chapter Three this dissertation). This suggests that only a small percentage of the bacterial population may be competent for adsorption. The sequential adsorption

experiments represented in Fig. 7 do not lend support to this hypothesis because similar numbers of A. tumefaciens cells bound to all three groups of discs. Mathysse and Gurlitz (1982) similarly found no change in the number of bacteria from the bacterial suspension when a 10-fold increase in plant cells was used.

Lippincott and Lippincott (1969) have proposed a specific site-attachment hypothesis to describe the adsorption of A. tumefaciens to cells of pinto bean leaves. When the virulent A. tumefaciens strain B6 was added to the wound prior to inoculation with the avirulent IIBNV6 strain, tumor number was not reduced. However, when the order of inoculation was reversed, a reduction in tumor number was noted. This hypothesis suggests a very specific irreversible adsorption of A. tumefaciens strain B6 to pinto bean leaf tissue. The data presented here suggest that A. tumefaciens adsorption is a nonspecific phenomenon governed by physisorption forces. If adsorption was site-specific and irreversible, equilibrium binding as is shown in Fig. 6 would not be expected. Reduced or elevated temperatures also would be expected to strongly influence an irreversable specific site-attachment.

However, there may be two separate types of binding sites. One could be site-specific and adsorb bacteria as a prelude to tumor formation. Other sites may nonspecifically adsorb bacteria and not lead to tumorigenesis. Since complete desorption of all bacteria from the potato surface during the 6 hr desorption period at 6°C was not obtained, the possible existence of two types of adsorption sites on potato tuber tissue varying in their affinity for Agrobacterium spp. cannot be eliminated. However, I did not obtain any direct evidence to support the existence of more than one type of adsorption site.

CHAPTER FIVE
EFFECTS OF AGENTS THAT ALTER IONIC AND HYDROPHOBIC INTERACTIONS ON AGROBACTERIUM TUMEFACIENS ADSORPTION

Introduction

The induction of tumors by A. tumefaciens is thought to require the site-specific attachment of the bacterium to a plant wound site (Lippincott and Lippincott 1969). Specific attachment was inferred from studies in which plants were inoculated with an avirulent A. tumefaciens strain prior to or coincident with a virulent A. tumefaciens strain. Changes in tumor number indicated that a single virulent bacterium attached to a single host site, and that such attachment resulted in a single tumor (Lippincott and Lippincott 1969, Whatley et al. 1977). If an avirulent cell occupied the site, the virulent cell was excluded, and a tumor did not form. Unfortunately, attachment was not quantified in these studies.

Various host and bacterial molecular components have been examined for their possible role in site-attachment. When bacterial suspensions were mixed with isolated plant cell wall fractions, tumor number was reduced (Lippincott and Lippincott 1977). Several off-the-shelf compounds were tested, and galacturonans were found to inhibit tumorigenesis dramatically. Galacturonans are thought to inhibit tumor formation by binding to the bacterial receptor that mediates attachment to the host. These results led to the hypothesis that a pectic component in the host's cell wall contains the specific attachment site for Agrobacterium sp. Crude lipopolysaccharides (LPS) and bacterial cell

envelope preparations from A. tumefaciens have been examined for their participation in specific-site attachment (Whatley et al. 1976). Plant tissue pretreated with the o-antigen region of the LPS reduces the ability of virulent bacteria to form tumors and thus is thought to mediate bacterial attachment to some pectic component in the plant cell wall.

Pueppke and Benny (1984) directly measured adsorption of A. tumefaciens to potato tissue. Adsorption was not reduced by galacturonans and in fact was enhanced by citrus pectin. Cell-free LPS from all but one strain did not influence bacterial adsorption. Additionally, LPS did not influence tumor formation in the potato tuber disc assay system (Pueppke and Benny 1983).

The heat of adsorption for A. tumefaciens attachment to plant cells is very small, an observation consistent with physisorption (Chapter Four this dissertation). Physisorption implies that adsorption is nonspecific and mediated by ionic and/or hydrophobic forces. Here I provided evidence to support the hypothesized nonspecific nature of A. tumefaciens adsorption. The involvement of ionic and hydrophobic forces in the adsorption process also was tested directly. The ionic nature of the bacterial surface was affected by changes in pH and divalent cations. The possible role of hydrophobic forces and bacterial proteins in adsorption also was tested.

Materials and Methods

Maintenance of Bacterial Strains

The sources of the Agrobacterium spp. and other bacteria used in this study are listed in Table 4. All of the strains were maintained on slants of the defined gluconate-mannitol medium of Bhuvaneswari et al. (1977)

Table 4. Sources of Characteristics of Bacterial Strains.

Strain	Source[a]	Type	Ti-plasmid	Pathogenic on Potato
A. tumefaciens				
B2F	Anand	Wild type	+	+
B6	Matthysse	Wild type	+	+
B6M	Anand	Laboratory substrain of B6	+	+
GV3132	De Block	Tn7 insertion mutant of pTi C58	+	+
178	Anand	Wild type	+	+
ATCC 15955	Gurley	Wild type	+	+
B6-95	Gurley	Avirulent B6 mutant	+	-
B2R	Pueppke	Spontaneous variant of B2F	+	+
NT1-B6	Gurley	NT1 transformed with pTi B6	+	-
ACH5	Lippincott	Wild type	+	+
C58	Lippincott	Wild type	+	+
ACH5C3	Lippincott	Heat-cured ACH5	-	-
NT1	Lippincott	Heat-cured C58	-	-
IIBNV6	Anand	Avirulent deviation of IIBV7	-	-
NT1-95	Gurley	NT1 transformed with pTi B6-95	+	-
Rhizobium japonicum				
USDA 201	Keyser	Fast grower nodulates soybean		
Xanthomonas vesicatoria				
71-21	Stall	Wild type		
A. radiobactor				
ATCC 4718	Anand	Wild type	-	-
S1005	Gurley	Wild type	-	-
K84	Lippincott	Wild type, Australia	-	-
A. rhizogenes				
15834	Lippincott	Wild type	Ri-plasmid	+
A rubi				
ATCC 13335	Anand	Wild type	+	+

[a] Sources of strains: Dr. V. K. Anand, Department of Biology, University of Missouri, St. Louis, MO 63121 U.S.A.; Dr. W. B. Gurley, Department of Microbiology and Cell Science, University of Florida, Gainesville, Fl 32611 U.S.A.; Dr. J. A. Lippincott, Department of Biological Sciences, Northwestern University, Evanston, IL 60201. U.S.A.; Dr. Ann G. Matthysse, Department of Botany, University of North Carolina, Chapel Hill, NC 27514 U.S.D.A.; Dr. S. G. Pueppke, and Dr. R. E. Stall Department of Plant Pathology, University of Florida, Gainesville, Fl 32611 U.S.A.; Dr. M. De Block, Rijksuniversiteit Gent, Ledeganckstraat 35, B-900 Gent, Belgium,

Bacterial Attachment Assay

One ml of buffered gluconate-mannitol medium (Meyer and Pueppke 1980), containing 50 uCi of ^{35}S-L-methionine, was inoculated with 1 to 2 X 10^7 bacterial cells. The culture was incubated for 15 to 16 hr on a rotary shaker at 125 rpm and 27°C. The bacteria were harvested by centrifugation at 10,000 X G for 10 min. The bacterial pellet then was washed three times with PBS. Specific activities ranged from 20 to 150 bacteria per cpm. The final bacterial concentration was adjusted turbidimetrically to 2 X 10^7 cells/10 ul of PBS.

Adsorption was measured by the potato tuber disc assay (Pueppke and Benny 1984) which is described here briefly. Potato tubers (Solanum tuberosum L. cultivar Red LaSoda or Red LaRouge) were washed, peeled, surface sterilized in a 20% solution of Clorox for 15 min and rinsed copiously with sterile water. A 8-mm diameter cork borer was used to extract a core of tissue, which was cut into 2-to 3-mm discs. The discs were blotted dry on filter paper and placed on a 10-ul droplet containing 2 X 10^7 radioisotope-labeled bacterial cells. After a 10 min adsorption period, the discs were washed with three 5-ml portions of PBS. Individual discs were transferred to glass scintillation vials, dried overnight, and digested with perchloric acid and hydrogen peroxide. Five ml of Amersham ACS II Scintillation fluid were added to each vial, and the radioactivity was determined with a Beckman LS-50 liquid scintillation spectrometer. The number of bound bacteria was estimated from the measured radioactivity.

Adsorption of A. tumefaciens to Monocot Tissue

Three monocot tissues were examined: 1) rhizomes of Smilax bona-nox L. (Liliaceae), 2) aerial tubers of Dioscorea bulbifora L. (Dioscoreaceae) and 3) corm tissue of Gladiolus tristis L. (Iridaceae). Each of these monocot tissues was prepared for the adsorption assay as described above. Dioscorea bulbifera and the Smilax bona-nox were collected near Gainesville, Fl. Gladiolus tristis corm tissue was kindly provided by Beth Logan, University of Florida.

Zeta Potential Measurements

Bacteria were washed twice with water, centrifuged at 7,700 X G for 10 min, and resuspended in one of the following buffers: 50 mM acetate buffer containing 7.2 g of NaCl/l (final pH 4.5), PBS (pH 7.2), or 50 mM borate buffer containing 7.2 g of NaCl/l (final pH 9.5). Measurements were made as described previously (Chapter Four this dissertation) with a Laser Zee Meter Model 502, set at 75 V. The zeta potential of the bacteria in the upper stationary phase was recorded.

Negative Staining for Electron Microscopy

Formvar-coated grids were sputter coated with carbon and placed on a bacterial colony growing on agar or floated on a bacterial suspension. After 30 min the grids were removed and washed thoroughly with water. Grids with adsorbed bacteria were stained with 1% phosphotungstic acid for 10 min and excess stain was removed by washing with water. The grids were dried and then viewed with a Hitachi-600 electron microscope.

Proteolytic Treatment of Bacteria

Radioisotope-labeled cells of A. tumefaciens strain ACH5 were adjusted to 1.6 X 10^9 cells/ml of PBS. A 12.5-ul droplet containing 2 X 10^7 cells was placed on a hydrophobic petri plate (Fisher No. 8-757-12), to which was added 12.5 ul of protease (Streptomyces griseus, Sigma Chem. Co.) at 1 mg/ml or trypsin (Bovine pancreas, Sigma Chem. Co.) at 250 mg/ml and incubated at room temperature for a predetermined time period. A potato disc (9-mm diam, 3-mm thick) then was placed onto the 25-ul droplet for a 10 min adsorption period. The number of bacterial cells bound to the surface was determined as described above. In replicate experiments, the disc size was reduced to 8 mm in diameter and the volume of the droplet was reduced to 20 ul.

Influence of $CaCl_2$ on Adsorption

A suspension of radioisotope-labeled bacterial cells was divided into two equal aliquots, each of which was washed separately. One aliquot was washed three times with 0.15 M NaCl (pH 7.2) and the other with a solution of 0.15 M NaCl (pH 7.2) containing 7.8 mM or 15.6 mM $CaCl_2$. The $CaCl_2$ treated suspension was adjusted to 2 X 10^7 cells/10 ul in the $CaCl_2$-NaCl solution and used for inoculation as described above. The control suspension was adjusted to 2 X 10^7 cells/10 ul in the NaCl solution. After the 10 min adsorption period, the discs were washed three times in the buffer originally used to wash the bacteria. Seven separate experiments were conducted with 10 replications in each.

Influence of a Calcium Chelating Agent on Adsorption

Potato discs were incubated in 3 mM aqueous ethylene-bis(oxyethylenenitrile) tetraacetic acid (EGTA) for 15 min at room temperature, after which the discs were removed and blotted dry on filter paper. Control potato discs were incubated in water for 15 min. The potato discs then were placed on a 10-ul droplet containing 2×10^7 radioisotope-labeled bacteria that had been washed five times with deionized water. After 10 min, the discs were treated as described above, and the number of bound bacteria determined. The experiment was repeated five times, with each experiment containing 10 replications.

Agglutination of Bacteria by Divalent Cations

Bacteria from 2-day-old cultures on glutamate-mannitol slants were washed twice, suspended in PBS, and adjusted to a concentration of 4.4×10^9 cells/ml. Fifty ul of a 1 M solution of $FeSO_4$, $CaCl_2$, or $MgSO_4$ were serially diluted in a two-fold series to a titer of 12 in a polystyrene V-type microtiter plate (Dyntech Laboratories, Alexandria, Va.). Twenty-five ul of PBS containing 1.1×10^8 cells were added to each well such that the final volume was 75 ul. The final concentration of Fe^{2+}, Ca^{2+}, or Mg^{2+} ranged from 670 mM to 0.33 mM. The microtiter plate was agitated gently and then left undisturbed for 3 hr at room temperature. The degree of agglutination was determined visually. The experiment was repeated twice, each with two replications.

Influence of Detergents on Adsorption

Bacteria were radioisotope-labeled as described above and washed four times with half-strength PBS. The bacterial suspension then was divided into four equal portions and centrifuged at 10,000 X G for 10 min. One of the four portions was resuspended in half-strength PBS (control) and the other portions resuspended in a half-strength PBS solution of the detergent to be tested. Three detergents were examined; sodium dodecyl sulfate (SDS) (anionic), hexadecyltrimethylammonium bromide (cationic), and Tween-20 (nonionic). The bacteria were incubated at room temperature in the detergent for 15 min, after which the detergent solution containing the bacteria was adjusted to a bacterial concentration of 2×10^7 cells/10 ul. The bacteria then were used as inoculum in the adsorption assay as described above. This experiment was conducted twice with 10 replications within each experiment.

Adsorption of A. tumefaciens to Hydrophobic Polystyrene

Bacterial adsorption to polystyrene was measured by three techniques. The first technique has been described previously (Rosenberg 1981), and is briefly outlined here. The bacteria to be examined were streaked onto glutamate-mannitol agar and incubated for 2 to 3 days at 27°C. A flat hydrophobic polystyrene disc, cut from the lid of a petri plate, was pressed against the surface of the bacterial colonies. After 5 to 6 min the plastic disc was removed and vigorously washed under a stream of tap water for approximately 2 min. The bacteria on the plastic surface were fixed by immersing the discs in methanol for 30 sec, after which they were air-dried, and stained with Gentian violet. A hair dryer then was used to dry the stained

plastic surface, containing the adsorbed bacteria. The plastic disc was immersed in tap water to selectively remove the stain from the plastic surface leaving the stained adsorbed bacteria. The intensity of staining was examined visually and the discs given a qualitative rating of ++ = high level of adsorption, + = medium level of adsorption, and - = no observable adsorption.

In the second technique, bacterial cells were radioisotope-labeled, washed as described previously, and adjusted to a final concentration of 2×10^7 bacteria/10-ul droplet of PBS. Discs (8 mm diam) were cut from the bottom of hydrophobic petri plates, and the discs were marked to ensure that the bacterial suspension would be applied to the untouched inside surface of the plastic disc. Ten ul of the bacterial suspension were placed on each plastic disc. After a 10 min adsorption period, discs were transfered using forceps to a beaker of water. The discs were agitated for 10 sec to remove both non- and loosely-adsorbed cells. Individual plastic discs were placed in scintillation vials, which contained 0.5 ml of a 4% aqueous solution of SDS. The vials were shaken to remove the adsorbed bacteria. Five ml of scintillation fluid were added to the vials, and radioactivity was determined.

To monitor the effect of pH on the adsorption of radioisotope-labeled bacterial cells onto hydrophobic polystyrene surfaces, the bacteria were washed and resuspended in either 50 mM acetate buffer containing 7.2 g of NaCl/l, (pH 4.5), PBS (pH 7.2), or 50 mM carbonate/bicarbonate buffer containing 7.2 g of NaCl/l (pH 9.5). The adsorption assay was carried out as described above. The experiment was repeated twice with 10 replications in each experiment.

The Effect of pH on Adsorption to Hydrophobic and Hydrophilic Plastic

A third adsorption technique was used to study the effect of pH on adsorption to both hydrophobic and hydrophilic polystyrene (Fletcher 1977).

Bacteria were washed three times with water, centrifuged at 8,000 X G for 10 min, resuspended in either acetate buffer (pH 4.5) PBS, (pH 7.2), or borate buffer (pH 9.5), and adjusted to a concentration of 5 X 10^8 bacteria/ml. Twenty ml of the bacterial suspension were poured into either a hydrophobic (Fisher No. 8-757-12) or hydrophilic (Corning No. 25020) 9-cm polystyrene petri plate. After 2 hr at room temperature the suspension was decanted, and the plates were gently washed with 30 ml of distilled water. The adsorbed bacteria then were fixed to the plastic surface by drying the surface thoroughly with a hair dryer. A freshly filtered solution of crystal violet solution (Conn 1940) was added until the bottom of the petri dish was covered. After 5 min the stain was decanted and the plates were slowly passed through a stream of running tap water five times to remove the excess stain. The plates were dried thoroughly with a hair dryer. Each petri plate was divided into eight equal sections by marking the edge with a felt pen. Absorbance values for each of the eight sections on the petri plate were obtained at 590 nm with Beckman ACTA CII spectrophotometer.

Results

Both monocot and dicot tissues are capable of adsorbing large numbers of cells of A. tumefaciens strain ACH5 (Table 5). The number of bacteria attached to S. bona-nox tissue after the 10 min adsorption period was significantly greater (P = 0.01) than that to D. bulfifera, G. tristis or S. tuberosum tissue. There was no significant difference in bacterial adsorption to D. bulbifera, G. tristis or S. tuberosum tissue (Table 5).

There was no significant difference in the capacity of ACH5 cells and any of the seven other bacterial species to bind to potato tissue (Table 6). However a significant difference does exist between two A. tumefaciens strains (ACH5C3 and IIBNV6) and A. radiobacter strain 4718 (Table 6). Included in the list is a fast-growing Rhizobium japonicum strain and Xanthomonas vesicatoria. Neither of these organisms is symbiotic or pathogenic on potato. A. tumefaciens GV3132, A. radiobacter 4718, and A. rhizogenes 15834 have been designated as non site-binding avirulent strains (Lippincott and Lippincott 1969). Their attachment to potato tuber tissue, however, was similar to that of the virulent site-binding strain ACH5.

Influence of Proteolytic Treatment of Bacteria on Adsorption

Preincubation of bacteria with protease for 30 min resulted in a statistically significant increase in the number of ACH5 cells bound relative to controls (Table 7). Trypsin treatment had no significant effect. A. tumefaciens B6 cells pretreated with either trypsin or protease bound in significantly greater numbers than did cells in the control.

Influence of Detergents on Adsorption

All three detergents influenced bacterial adsorption to potato tissue, but the ionic character of the detergent does not appear to be an important factor. Tween-20 at concentrations of 7%, 0.4%, and 0.04% (v/v) inhibited

Table 5. Adsorption of Agrobacterium tumefaciens ACH5 to Monocot and Dicot Tissue.

Plant Species	Number of Bacteria Bound per Disc ($X\ 10^5$)*	
Smilax bona-nox	86.0 ± 18.2 a	Monocot
Dioscorea bulbifera	18.3 ± 09.0 b	Monocot
Gladiolus tristis	16.0 ± 06.7 b	Monocot
Solanum tuberosum	8.3 ± 02.3 b	Dicot

The values are means (±1 standard deviation) of three experiments, each of which contained 10 replications.
*Numbers followed by the same letter are not significantly different (P = 0.05) according to Duncan's Multiple Range Test.

Table 6. Adsorption of Bacteria to Potato Tuber Tissue

Bacterial Species	Average Number of Bacteria Bound per Disc (X 10^5)
Agrobacterium tumefaciens ACH5	5.10 ± 2.8 ab*
Agrobacterium tumefaciens ACH5C3	9.76 ± 1.7 a
Agrobacterium tumefaciens IIBNV6	8.98 ± 1.6 a
Agrobacterium tumefaciens GV3132	6.41 ± 1.0 ab
Agrobacterium rhizogenes 15834	4.40 ± 2.1 ab
Agrobacterium radiobacter 4718	1.70 ± 0.4 b
Rhizobium japonicum USDA 201	7.85 ± 1.5 ab
Xanthomonas vesicatoria 71-21	6.88 ± 2.6 ab

The values are means (±1 standard deviation) of two experiments, each of which contained 10 replications.
*Numbers followed by the same letter are not significantly different (P = 0.05) according to Duncan's Multiple Range Test.

Table 7. Influence of Proteolytic Enzymes on the Binding of Agrobacterium tumefaciens Strains ACH5 and B6 to Potato Tissue.

Treatment	ACH5		B6	
	Mean No. Cells Bound per Disc (X 10^5)	% of Control	Mean No. Cells Bound per Disc (X 10^5)	% of Control
Protease	10.05 a*	142	16.71 a	356
PBS	7.06 b	100	4.69 b	100
Trypsin	6.16 b	87	13.83 c	294

Each value is the mean of eight replications.
*Numbers followed by the same letter are not significantly different (P = 0.05) according to Duncan's Multiple Range Test.

bacterial adsorption by 56%, 41% and 20%, respectively. In the presence of 0.05% Tween-20, adsorption was reduced from $2.8 \pm 0.4 \times 10^6$ to $1.87 \pm 0.4 \times 10^6$ cells/disc, a 35% reduction. Adsorption was reduced from $2.80 \pm 0.5 \times 10^6$ cells bound/disc in the control to $0.95 \pm 0.3 \times 10^6$ cells bound/disc by 0.05% SDS. This is a 66% reduction in the number of cells bound. Adsorption was reduced by 35%, from $2.8 \pm 0.5 \times 10^6$ to $1.82 \pm 0.27 \times 10^6$ bacteria per disc, in the presence of 0.5% hexadecyltrimethylammonium bromide as compared to the control. Tween-20 and SDS did not reduce bacterial colony forming units (CFU) at the concentrations studied, whereas 0.05% hexadecyltrimethylammonium bromide reduced bacterial CFU.

Adsorption of Agrobacterium spp. to Hydrophobic Polystyrene

Ten strains of A. tumefaciens, one A. rhizogenes strain, two A. radiobacter strains, and one A. rubi strain were screened semiquantitatively for their ability to adsorb to hydrophobic polystyrene (Rosenberg 1981). Both virulent and avirulent Agrobacterium spp. adsorb to hydrophobic polystyrene (Table 8). Significantly, strain B2R, a spontaneous mutant of B2F, is able to adsorb to hydrophobic plastic, but B2F is not.

Influence of pH on Bacterial Adsorption to Plastic

There was no significant effect of pH on adsorption of ^{35}S-labeled A. tumefaciens ACH5 to hydrophobic plastic. The numbers of cells bound per disc were $1.3 \pm 0.45 \times 10^6$, $1.2 \pm 0.6 \times 10^6$, $0.95 \pm 0.3 \times 10^6$ at pH 4.5, 7.2, 9.5, respectively. When measured photometrically, the adsorption to hydrophobic

Table 8. Adsorption of Agrobacterium spp. to Hydrophobic Polystyrene.

Agrobacterium spp.		Adsorption*
A. tumefaciens	B6	++
	B6M	++
	C58	++
	ACH5	++
	B2R	+
	1400	+
	15955	+
	B6 - 95	+
	NT1	+
	B2F	-
	NT1(95)	-
A. rhizogenes	15834	-
A. radiobacter	S1005	+
A. rubi	13335	-

*Adsorption is based on a visual, semi-quantitative rating:
++ = high level of adsorption, + = medium level, and - = no visually observable adsorption

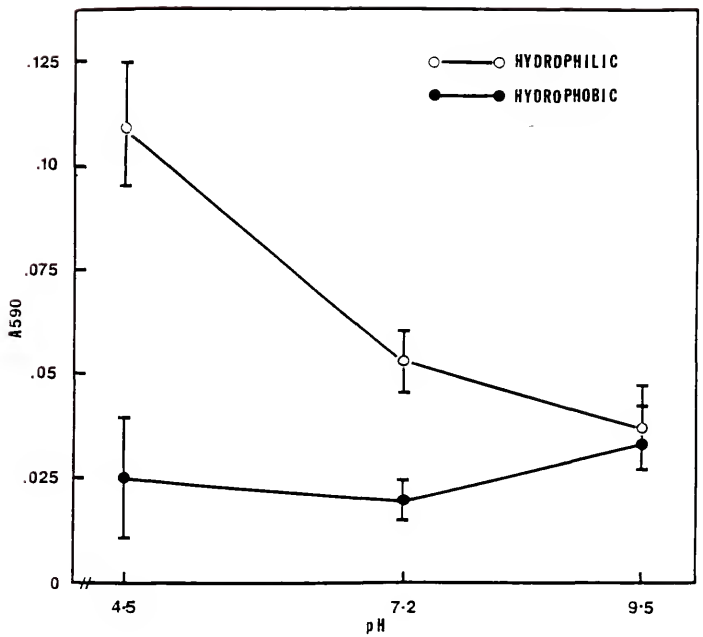


Fig. 9. Adsorption of Agrobacterium tumefaciens strain ACH5 to hydrophilic or hydrophobic polystyrene as a function of pH. Bars represent ±1 standard deviation.

plastic also was not significantly influenced by pH (Fig. 9). Adsorption to hydrophilic plastic, however, was significantly greater at pH 4.5 than at pH 7.2 or pH 9.5 (Fig. 9).

Adsorption of A. tumefaciens GV 3132 to potato tuber discs was optimal at pH 7.2 ($6.5 \pm 0.95 \times 10^5$ bacteria/disc) and significantly reduced at both pH 4.5 ($1.72 \pm 0.38 \times 10^5$ bacteria/disc) and pH 8.5 ($2.1 \pm 0.75 \times 10^5$ bacteria/disc) according to the Student's t-test.

Effect of EGTA Treatment

Pretreatment of potato discs with 3 mM EGTA did not influence their ability to adsorb radioisotope-labeled bacteria. Discs treated with EGTA were compared with PBS control discs and analyzed using the Student's t-test. One-sided tests at the P = 0.05 level indicate that treatment of the potato discs with EGTA does not have a significant effect on bacterial adsorption. In these experiments pretreated EGTA discs bound $1.41 \pm 0.56 \times 10^6$ bacteria per disc, and control discs bound $1.54 \pm 0.80 \times 10^6$ bacteria disc (± 1 standard deviation).

Effect of Divalent Cations on Agglutination

A. tumefaciens ACH5 cells are agglutinated by $CaCl_2$. The dilution end point is 5.2 mM. The greatest agglutination of ACH5 occurred at a $CaCl_2$ concentration of 83 mM. $FeSO_4$ exhibited a tremendous ability to agglutinate A. tumefaciens. Agglutination was evident in the last well, which contained 0.33 mM $FeSO_4$. The greatest degree of agglutination was found in the presence of 5.2 mM $FeSO_4$.

Even after 6 hr in the presence of 1 M $MgCl_2$, agglutination of ACH5 was slight. However, in the presence of either $CaCl_2$ or $FeSO_4$ agglutination was very rapid and nearly complete within 1 hr, and stable overnight at room temperature. Control bacteria suspended in either water or PBS did not agglutinate.

Effect of $CaCl_2$ on Adsorption to Plant Tissue

Binding of strain ACH5 to potato tissue was not appreciably affected by 7.8 mM or 15.6 mM $CaCl_2$ (Table 9). Comparison of $CaCl_2$-treated to non-treated controls by the Student's *t*-test showed no significant difference in the number of bacteria bound. However, in six of the seven experiments, potato discs consistently bound fewer $CaCl_2$-treated bacteria. The $CaCl_2$ concentrations used in these experiments fall near the dilution end point of agglutination. Therefore, agglutination of the bacteria during washing and the subsequent binding period should have been minimal.

Zeta Potential Measurements

The zeta potential of bacteria suspended in the borate buffer (pH 9.5) is -11 mV. Bacteria suspended in PBS have a zeta potential of approximately -2.5 mV. In acetate buffer (pH 4.5) the zeta potential is slightly positive with a value of +0.1 mV. It should be emphasized that zeta potential determinations are more variable at or near the isoelectric point of the particle. Therefore

Table 9. Effect of $CaCl_2$ on Agrobacterium tumefaciens Adsorption.

Experiment	$CaCl_2$ (mM)	Cells bound (X 10^6)		Statistical Significance
		$CaCl_2$	Control	
1	7.8	1.40 ± 0.63	1.80 ± 0.49	NS
2	7.8	1.15 ± 0.25	1.30 ± 0.74	NS
3	7.8	1.60 ± 0.80	1.98 ± 0.40	NS
4	15.6	0.97 ± 0.10	0.80 ± 0.20	NS
5	15.6	1.20 ± 0.38	1.50 ± 0.39	NS
6	15.6	0.70 ± 0.20	1.00 ± 0.19	NS
7	15.6	0.86 ± 0.26	0.90 ± 0.22	NS

All adsorption experiments were carried out with an initial inoculum of 2 X 10^7 bacteria/ml.
Statistical significance was determined with the Student's t-test. (P = 0.05).

the values given for measurements at pH 4.5 and pH 7.2 are not necessarily the absolute zeta potentials of the bacteria suspended in either of those buffers.

Negative Staining

The result of negative staining cells of strain ACH5 with phosphotungstic acid is shown in Fig. 10. Bacteria from either colonies on agar plates or suspension cultures gave similar results (Fig. 10).

Discussion

The transfer of the T-DNA from Agrobacterium spp. into the host cell most likely requires the attachment of the bacterium to the plant cell surface. The bacterial o-antigen and the pectic materials in the host cell wall have been hypothesized to mediate such bacterial adsorption (Lippincott and Lippincott 1969, Whatley et al. 1976, Lippincott et al. 1977b, Rao et al. 1982). These data, however, should be viewed with caution, because bacterial adsorption was not measured directly (Lippincott and Lippincott 1980). Pueppke and Benny (1981, 1983) have shown that extrapolation from tumor formation to bacterial adsorption may not be justified. Additionally, Pueppke and Benny (1983) used the potato tuber disc assay to show that neither tumor formation nor A. tumefaciens adsorption was inhibited by cell-free LPS. In contrast, inoculation of pinto bean leaves in the presence of cell-free LPS substantially reduced tumor formation (Whatley et al. 1976).

The site attachment hypothesis predicts that the relative resistance of most monocotyledonous plants to Agrobacterium spp. is a consequence of their

Fig. 10. Negatively stained Agrobacterium tumefaciens strain ACH5. X 10,000.

inability to adsorb Agrobacterium spp. (Lippincott and Lippincott 1969, Lippincott et al. 1977b, Lippincott and Lippincott 1978a). However, adsorption of virulent, site-binding A. tumefaciens cells to S. tuberosum, was not significantly different (P = 0.05) than that to two monocots, D. bulbifera, and G. tristis. However, the number of bacteria bound to the S. bona-nox tissue was significantly greater than that bound to any of the other species. Ohyama et al. (1979) have shown that suspension culture cells of a variety of monocots adsorbed large numbers of A. tumefaciens cells. In addition, suspension culture cells of soybean (Glycine max) bound a large number of A. tumefaciens cells, even though soybean apparently is resistant to A. tumefaciens infection (De Cleene and De Ley 1976). Draper et al. (1983) have shown that suspensions of cells of another monocot, asparagus, are agglutinated by A. tumefaciens cells.

A series of virulent and avirulent Agrobacterium spp. adsorb in statistically similar numbers to potato tuber tissue. The adsorption of all of these species is similar to that of A. tumefaciens ACH5 (Table 6). Additionally, the adsorption of Rhizobium japonicum USDA 201 and Xanthomonas vesicatoria 71-21 were not significantly different from that of strain ACH5 (Table 6).

Divalent calcium did not statistically alter the number of bacteria bound to wounded tissues. Additionally, when discs were treated with the calcium chelating agent EGTA prior to bacterial inoculation, no alteration of bacterial adsorption was observed. This suggests that the availability of Ca^{2+} is not important to bacterial adsorption. Ohyama et al. (1979) reported somewhat similar results using suspension cultured cells.

In light of a report suggesting that LPS and galacturonans do not participate in A. tumefaciens adsorption to susceptible potato tissue (Pueppke

and Benny 1984), the actual molecular components of A. tumefaciens adsorption remain unclear. A. tumefaciens adsorption, however, may be described in terms of physical adsorption, which is governed by ionic and hydrophobic forces (Chapters Three and Four this dissertation). The adsorption of A. tumefaciens ACH5 to hydrophobic plastic was not altered as a function of pH. However, adsorption to hydrophilic plastic was optimal at pH 4.5, and decreased with increasing pH (Fig. 9). When measured as a function of pH, adsorption of A. tumefaciens GV3132 to potato tuber tissue was optimal at pH 7.2, and significantly decreased at either acidic or basic pH. Similarly, Pueppke and Benny (1984) observed that the adsorption of A. tumefaciens ACH5 to potato tuber tissue was optimal at pH 7.2 and reduced at either pH 4.5 or 9.5. Zeta potential measurements of the bacteria suspended in the same buffers, show that the surfaces of the bacterial cells are highly negatively charged at pH 9.5 (borate), are neutral to slightly negative at pH 7.2 (PBS), and are slightly positive at pH 4.5 (acetate buffer).

As the pH approaches the pI of A. tumefaciens cells, a reduced surface charge may facilitate a closer approach to the host's surface. Attachment then may be facilitated by cellular appendages, such as the flagellae shown in Fig. 10. These could help overcome the remaining electrostatic repulsion barrier between the bacterium and the plant cell (Ottow 1975). Matthysse et al. (1982) illustrated the adsorption of A. tumefaciens in large numbers to the cell membranes of tobacco and carrot protoplasts that were devoid of cell wall material. The importance of hydrophobic interactions in the adsorption process that leads to tumor formation remains to be determined. It is clear, however, that not all Agrobacterium spp. adsorb to hydrophobic plastic similarly, as measured according to Rosenberg (1981) (Table 8), and yet are able to adsorb to potato tuber tissue (Table 6).

The zeta potential of bacteria suspended in deionized water at pH 4 or greater is highly negative (Chapter Four this dissertation), yet in acetate buffer at pH 4.5, the zeta potential is slightly positive. The descrepancy can be explained as follows. Bacteria suspended in the acetate buffer pH 4.5 adsorb cations from the solution onto their surfaces. This alters their behavior in an electrical potential and results in a less negative zeta potential. The effect is even more dramatic with bacteria suspended in PBS at pH 7.2. In water (low ionic strength) the bacteria normally would be negatively charged at pH 7.2. However, in PBS the negative bacterial surfaces adsorb cations from the solution, and this substantially reduces the negative zeta potential.

Outer membrane proteins exposed to the environment mediate hydrophobic interactions in several mammalian systems (Berkeley et al. 1980, Bitton and Marshall 1980). Pretreatment of A. tumefaciens with either trypsin or protease to digest exposed protein components resulted in increased numbers of bacteria bound. Consequently, the presence of intact outer proteins may in some way inhibit adsorption. Treatment of the bacteria with proteolytic enzymes presumably degrades external protein components, outer membrane proteins, pili, and flagellae, all of which may allow a closer approach of the bacterium to the host's cell surface. At this point the much shorter range of ionic forces comes into play, perhaps accounting for the increased adsorption observed after proteolytic digestion (Table 7). Alternatively, the enzymes themselves may adsorb tenaciously to the bacterial surface, enhancing the adsorption capacity of the bacteria.

At a concentration of 0.05%, all three detergents substantially reduced bacterial adsorption. The sensitivity of adsorption to the presence of detergents suggests that hydrophobic interactions play a role in A. tumefaciens

adsorption to potato tissues. Pili are a likely external component of the bacterium that could be involved in hydrophobic interactions. For example, gonococcal pili have been shown to be very hydrophobic with approximately 46% of the amino acid resides non-polar. This causes the pili to exist in the aggregated form in vitro. Characterization of Agrobacterium spp. pili and examination of their involvement in the adsorption process will certainly enhance our understanding of the attachment process.

These results, in addition to the fact that the genus Agrobacterium has the largest host range of any known phytopathogenic prokaryote, do not lend support to the site-attachment hypothesis, which describes a highly specific adsorption of Agrobacterium spp. to the surface of plant tissue. Rather, a nonspecific adsorption mediated by physical adsorption forces appears to be operative in the potato tuber disc assay system. Adsorption to plant and plastic surfaces is pH-sensitive. Anionic, cationic, and neutral detergents significantly reduce the number of A. tumefaciens adsorbed to plant cell surfaces. These results strongly indicate that both ionic and hydrophobic forces are involved in the non-specific adsorption of A. tumefaciens to plant cell surfaces.

CHAPTER SIX
SCANNING ELECTRON MICROSCOPY OF POTATO TUBER TISSUE INOCULATED WITH AGROBACTERIUM TUMEFACIENS

Introduction

The etiological agent of crown gall disease Agrobacterium tumefaciens, causes a tumor-like growth on many dicotyledonous plants (De Cleene and De Ley 1976). Once in a wound site on the plant, A. tumefaciens cells are thought to adsorb to a surface component of the host cell. A piece of the bacterial Ti-plasmid, known as the T-DNA, is subsequently transferred to the host, where it is stably incorporated and expressed (Watson et al. 1975, Chilton et al. 1977, Gurley et al. 1979, Thomashow et al. 1980). The method of DNA transfer, however, is unknown.

Plant protoplasts have been transformed by both intact A. tumefaciens cells and isolated Ti-plasmid (Marton et al. 1979, Wullems et al. 1981, Krens et al. 1982, Hanold 1983). It is possible that a similar situation occurs in a plant wound site, where the partial removal of cell wall material could expose the intact cytoplasmic membrane. This may facilitate the transfer to T-DNA out of the bacterium into the wounded host cell. A second possibility is that of bacterial-plant conjugation (Roberts and Kerr 1974, Kerr 1975). A third alternative is that intact bacteria penetrate viable plant cells and then transfer T-DNA. This idea has been rejected by some (Stonier 1956, Schilperoort 1969) and proposed as the essential step in tumorigenesis by others (Sigee et al. 1982).

The purpose of this research was to examine the infection of potato tissue by A. tumefaciens at the ultrastructural level. An avirulent, heat-cured strain was included for comparison.

Materials and Methods

Bacterial Culture, Maintenance, and Inoculum Preparation

Agrobacterium tumefaciens strains ACH5, ACH5C3, and B6 were maintained at 4°C on slants of the defined gluconate-mannitol medium of Bhuvaneswari et al. (1977). Inocula were prepared by streaking A. tumefaciens cells onto gluconate-mannitol slants. After 2 to 3 days at 27°C, the cells were washed from the slants with sterile PBS, centrifuged for 10 min at 7700 X G, and washed twice with PBS. The bacteria then were resuspended in PBS and adjusted turbidimetrically to a concentration of 2×10^7 cells/ml.

Preparation of Protoplasts

Suspension culture cells derived from potato (Solanum tuberosum cv Red LaSoda) tubers were maintained in Murashige and Skoog media (Gamborg and Wetter 1975). Cells were harvested and suspended in an equilibration medium consisting of B5 salts (Gamborg and Wetter 1975), 30 mM MES (2 N-morpholino ethanesulfonic acid), and 300 mM sorbitol, adjusted to pH 5.6. This suspension was incubated at 150 rpm at room temperature. After equilibration for 1 hr, the cells were harvested by centrifugation at 1000 X G for 10 min and resuspended in an enzyme mixture, where they were incubated for 4-6 hours at room temperature at 150 rpm. The enzyme solution contained 250 mg of

cellulase (Calbiochem, La Jolla, CA), 50 mg of Macerase (Calbiochem), 250 mg of Rhozyme (Rohm and Haas), and 50 mg of Driselase (Sigma) dissolved in 10 ml of the equilibration medium used above. After enzyme digestion the cell suspension was centrifuged at 30 X G rpm for 10 min and the protoplasts removed from the surface of the enzyme solution. The protoplasts were then washed three times with the equilibration medium.

Inoculation Procedure

Potato tuber discs were inoculated with A. tumefaciens as described by Pueppke and Benny (1981). Peeled potato tubers are surface sterilized for 10 min, and 8-mm-diameter cores of tuber tissue are removed. Discs, 2 to 3 mm thick, are sliced from the cores and immersed in a suspension containing 2 X 10^7 cells of strain ACH5 or ACH5C3/ml of PBS. A ratio of 1 ml of bacterial suspension per disc was maintained. After 10 min the discs were removed, rinsed for 2 or 3 seconds in sterile PBS, blotted dry, and placed on 1.5% water agar plates and stored in the dark at 28°C. At specified times after inoculation, discs were removed for scanning electron microscopy or the determination of bacterial colony forming units. The number of bacteria colonizing the potato disc was determined as described previously (Chapter Three this dissertation).

Isolated protoplasts were inoculated with A. tumefaciens strain B6 at a concentration of 2 X 10^7 cells/ml. After incubation for 3 hr the inoculation mixture was filtered through Whatman No. 1 filter paper, and the protoplasts rinsed with two volumes of equilibration buffer. The filter paper, which then contained the protoplasts and bound bacteria, was removed and prepared for scanning electron microscopy.

Fixation for Scanning Electron Microscopy

Discs were placed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2% glutaraldehyde and fixed overnight at 6°C. Samples then were rinsed three times with 0.1 M cacodylate buffer and post-fixed overnight at 6°C in 1% OsO_4. The discs then were rinsed and sequentially dehydrated in solutions of 25%, 50%, 75%, 95%, and 100% ethanol. Samples were transferred into fresh 100% ethanol, which was changed twice, and then dried in a CPD 010 critical point drying apparatus using CO_2 as the exchange fluid. Specimens then were sputter coated with gold and viewed with a Hitachi S-450 scanning electron microscope using an accelerating voltage of 20 KV. Discs were fixed for scanning electron microscopy at time intervals of 0, 1, 2, 6, 8, 19, and 24 hr post-inoculation and then every 24 hr up to 12 days after inoculation.

To prevent lysis of the protoplasts during fixation, 2% glutaraldehyde and 1% OsO_4 were prepared in the equilibration medium. Three extra steps in the ethanol dehydration series (35%, 65%, and 85%) were included to minimize artifacts caused by rapid dehydration.

Results

The cut surface of potato tubers provides a very heterogenous substrate for adsorption of A. tumefaciens cells. Cut cell wall fragments often create cavities, the bottoms of which may be covered with cytoplasmic membranes or tonoplasts of the ruptured cells or the outer walls of the cells beneath. Granules of various sizes are found dotting the surface. A. tumefaciens cells

are bound to broken cell wall fragments, membranous components, starch granules, etc., with no apparent affinity for any particular component (Fig. 15, 16, 17).

The adsorption of A. tumefaciens cells to the potato disc surface was examined during two different time courses in these experiments. The first time course extended from inoculation to 24 hr post-inoculation and is termed the early events time period. Although the bacterial inoculum contained only normal rod-shaped bacteria of approximately 2 to 3 um X 0.7 um in size (data not shown), bacterial rods are rarely found on the surfaces of inoculated potato discs until 6 to 8 hr post-inoculation. Instead, spherical structures, 0.5 to 1.5 um in diameter, are present on the disc surface (Fig. 11, Fig. 12). A field of view approximately 900 um^2 was maintained when scanning the disc surface. Assuming random distribution of the 2 X 10^5 bacteria that adsorbed per disc (8 mm dia X 2.2 mm thick), 1.3 bacteria should be in each field. Twenty to thirty fields, however, were examined before a single rod-shaped bacterium was observed in samples harvested before 6 hr post-inoculation. The bacterial rods were apparent in nearly every 900-um^2 field of samples taken 6-8 hr after inoculation (Fig 13).

The second time course, termed the late events time period, compared the behavior of pathogenic and nonpathogenic A. tumefaciens strains on inoculated potato tuber tissue 1 to 12 days after inoculation. Few differences are observed in the surface ultrastructure of discs inoculated with virulent A. tumefaciens ACH5 or the heat-cured, avirulent derivative, ACH5C3. Most noteworthy is the appearance of tumors at day 5 on tissues inoculated with ACH5 (Fig 22), but not on those inoculated with ACH5C3. Both strains formed

microcolonies. Incipient microcolonies are first noted 8 hr after inoculation (Fig. 13) and develop into dome-shaped structures within 19 hr (Fig. 14). The microcolonies initially are small and appear to continually increase in size. New microcolonies appear to be initiated over a long time period, because small domed colonies are present in samples harvested on day 12. Based on data from plating experiments bacterial multiplication on each disc is roughly equivalent for both strains and increases during an 8-day time period (Table 10). These colonies consist of densely packed bacteria apparently held together by some type of matrix (Fig. 15, 16, 17). For example, in Fig. 15 approximately 300 bacteria are tightly packed into a dome-shaped structure 6.5 um in diameter. Unique microcolony shapes are not associated with any particular stage of colony development. Microcolonies often are dome-shaped, but they also can be elongate and span 40-50 um (Fig. 15, 16, and 17).

Fig. 18 illustrates another point of interest. Rod-like structures appear to originate from the surfaces of both virulent and avirulent A. tumefaciens cells and to anchor individual bacterial cells to plant cell surfaces. These structures are seen as early as 24 hr and as late as 12 days post-inoculation. However, cellulose microfibrils were never observed in this study.

The adsorption of A. tumefaciens strain B6 to protoplasts from potato suspension cultures also was examined (Fig. 19, 20, 21a, 21b). Although agglutination of the plant cells had occurred at the time of fixation, 3 hr post-inoculation (Fig. 21a), cellulose microfibrils are not evident, and microcolony formation is not observed at this early time after inoculation. Most noteworthy, however, is the predominance of polar attachment, which is a rare event on potato tuber tissue (Fig. 19, Fig. 20).

Table 10. Bacterial Colonization of Potato Tuber Discs.

Time After Inoculation	Agrobacterium tumefaciens strain (cells/disc)*	
	ACH5	ACH5C3
0 hr	$1.8 \pm 0.35 \times 10^5$	$2.1 \pm 0.80 \times 10^5$
1 hr	$1.3 \pm 0.28 \times 10^5$	--
2 hr	$1.7 \pm 0.19 \times 10^5$	--
8 hr	$7.7 \pm 0.57 \times 10^5$	--
20 hr	$3.9 \pm 1.40 \times 10^7$	--
1 day	$3.1 \pm 0.60 \times 10^8$	$5.1 \pm 0.90 \times 10^8$
2 days	$2.8 \pm 0.90 \times 10^9$	$1.4 \pm 0.34 \times 10^9$
3 days	$1.1 \pm 0.75 \times 10^{11}$	$1.3 \pm 0.72 \times 10^{11}$
8 days	$2.0 \pm 0.30 \times 10^{11}$	$2.1 \pm 0.10 \times 10^{11}$

* The values are means ($\pm$1 standard deviation) of two experiments.

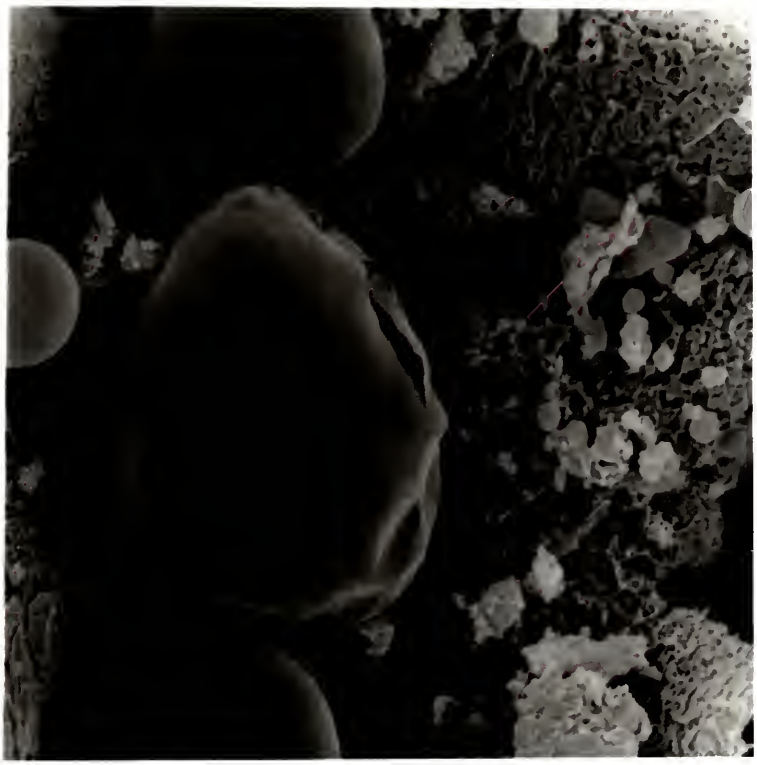


Fig. 11. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (10 min after inoculation). Note the presence of a large number of spherical structures approximately 0.9-1.5 um in diameter. No rod-shaped bacteria are present in this field of view. Three large starch particles are illustrated, and the central particle is ruptured. X 4,000.

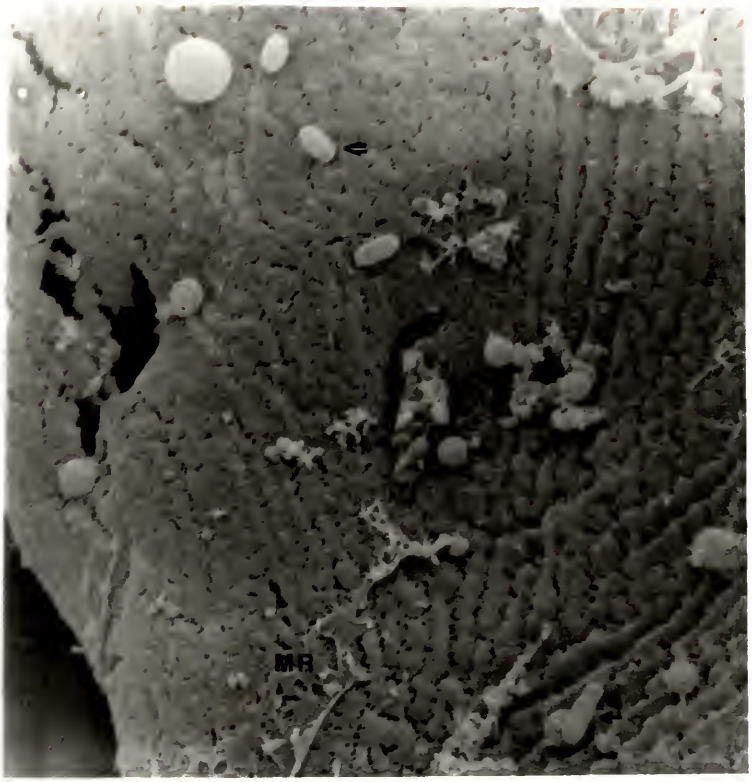


Fig. 12. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (1.5 hr after inoculation). The plant cell surface appears to be rippled and contains material that may be remnants of a membrane (MR). One rod-shaped bacterial cell is present (single arrow). Double arrows identify short rods and spherical structures that appear to be pleomorphic bacteria. X 7,600.

Fig. 13. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (8 hr after inoculation). Three rod-shaped bacteria are in the initial stages of microcolony formation. Note the ring of material around and on the bacteria. This material probably consists of dehydrated polysaccharide. X 34,000.

Fig. 14. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (19 hr after inoculation). Ten to twelve bacteria are forming an incipient microcolony (upper center of photograph). X 8,500.

Fig. 15. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5C3 (3 days after inoculation). Note the different morphology of the microcolonies: an elongated colony on the right and a tightly packed dome-shaped colony on the left of the photograph. X 5,300.

Fig. 16. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (8 days after inoculation). Both elongated and dome-shaped colonies are present. The elongated microcolony in the upper right corner (arrow) appears to be ruptured. The bacteria are also colonizing the area around the two starch grains (SG) located below the ruptured colony. Colonies of the size and shape seen in this field of view dot the entire surface of the disc. X 1,800.

Fig. 17. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5C3 (4 days after inoculation). Note the torn wall fragment (WF) in the upper right and the adsorbed bacteria (arrow). Microcolonies and many single adsorbed bacteria are scattered across the surface. X 3,200.

Fig. 18. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (7 days after inoculation). Note the rod-shaped structures (arrows) connecting the bacteria to the potato disc surface and to each other. X 7,600.

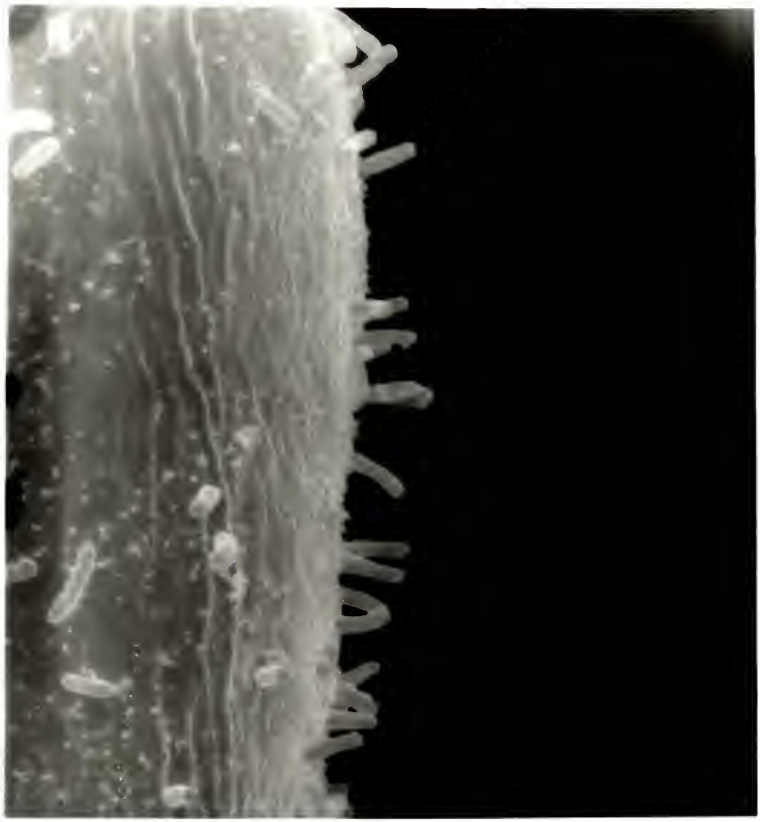


Fig. 19. Suspension culture potato protoplast inoculated with Agrobacterium tumefaciens strain B6 (3 hr after inoculation). The polar attachment viewed here was common. Recumbent attachment of the bacterium to protoplasts was observed infrequently. Note the absence of cellulose microfibrils. X 8,800.

Fig. 20. Suspension culture potato protoplasts inoculated with Agrobacterium tumefaciens strain B6 (3 hr after inoculation). Note polar attachment of bacteria to the protoplast surface and the absence of cellulose microfibrils. X 6,400.

Fig. 21. Suspension culture potato protoplasts inoculated with Agrobacterium tumefaciens strain B6, 3 hr after inoculation. a) Strings of bacteria have tied the protoplasts together resulting in agglutination. Note the polar attachment of the bacteria to the protoplast. X 2,500. b) Enlargement of the bacterial string shown in Fig 21a (boxed area). Note the absence of cellose microfibrils. X 6,200.

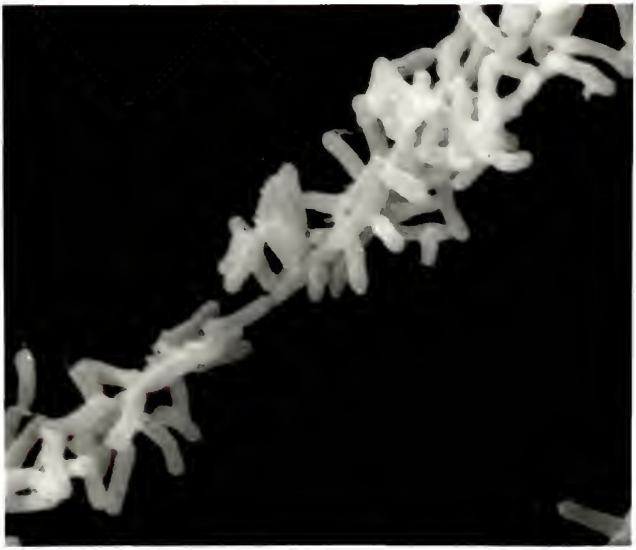


Fig. 22. Potato disc inoculated with Agrobacterium tumefaciens strain ACH5 (5 days after inoculation). Lateral view of tumor fills the photograph. X 400.

Discussion

The initial step in tumor formation is thought to be the adsorption of A. tumefaciens cells to the host cell surface (Lippincott and Lippincott 1969). Such adsorption may facilitate the transfer of the T-DNA (Lippincott and Lippincott 1969, Matthysse et al. 1981, Matthysse 1983), and thus adsorption may be a regulatory step in tumorigenesis. The basis of the resistance of monocots to A. tumefaciens is thought to lie in the inability of A. tumefaciens cells to adsorb, thereby preventing the transfer of the T-DNA (Lippincott and Lippincott 1978, 1980). Subsequently, however, workers have shown by direct measurement that both tissue and suspension culture cells from monocotyledonous plants are able to adsorb Agrobacterium spp. in substantial numbers (Ohyama et al. 1979, Chapter Three this dissertation). Additionally, equivalent numbers of both avirulent and virulent Agrobacterium spp. bind to potato tissue (Chapter Three this dissertation). Therefore, it was of interest to examine not only adsorption, but also post-adsorption events that might differentiate avirulent from virulent A. tumefaciens strains.

Parallel time-course experiments with the virulent strain ACH5 and its heat-cured avirulent derivative gave similar results. Cellulose microfibrils, which have been reported to anchor cells of A. tumefaciens strain A6 to suspension culture cells and protoplasts of carrot (Matthysse et al. 1981, Matthysse 1983) were not seen. The cellulose microfibrils appear within 2 hr of coincubation with carrot protoplasts and are suggested to participate in the firm attachment of large numbers of bacteria to the host cell (Matthysse 1983). Similarly, Spiess et al. (1977a, 1977b) observed a dense fibrillar matrix, apparently of bacterial origin, that entrapped A. tumefaciens cells bound on the surface of moss (Pylaisiella selwynii).

After a 3-hr coincubation of potato tuber protoplasts with A. tumefaciens strain B6, massive agglutination of the protoplasts had occurred, even though microfibrils were not observed. Two features may account for the absence of microfibrils in this system, and their presence on carrot protoplasts. The host tissue and A. tumefaciens strains are different in each of these experiments, as is the inoculation procedure. The inoculation period for potato tuber tissue was 10 min, after which the discs were rinsed, blotted dry, and placed on water agar plates. In both the carrot suspension cell and moss cell systems, coincubation lasted 2 to 19 hr.

Most A. tumefaciens cells were oriented perpendicularly on the protoplast surface, but not on the potato tuber surface. Habituated tobacco callus cells also adsorbed A. tumefaciens in a polar orientation (Smith and Hindley 1978). Such an orientation, however, is of questionable significance. Members of the closely related genus, Rhizobium, adsorb perpendicularly to all sorts of surfaces (Pueppke 1984). Marshall and Cruickshank (1973) and Marshall et al. (1975) observed that rhizobia orient themselves at right angles to oil-water interfaces, to blocks of Spurr's medium, and to legume root hair surfaces. This suggests that polar attachment of bacteria to surfaces may be nonspecific.

During the early time course, from time 0 to 6 hr after inoculation, normal bacterial rods were extremely difficult to find on the surfaces of potato discs. Spherical structures with dimensions similar to those of bacteria were common at this time. By 6 hr post-inoculation normal rod-shaped bacteria were observed in nearly every field of view. This suggests that the bacteria may be pleomorphic after introduction into a plant wound site. Fixation artifacts are not likely to be responsible for the pleomorphic structures, because discs harvested 12 hr after inoculation and at time 0 were fixed and

dried for SEM simultaneously and under identical conditions; normal rod-shaped bacteria were abundant in the former, but very rare in the later. Identification of the spherical particles is complicated by the presence of many spherical starch grains in the preparations. A. tumefaciens cells are, however, capable of forming spherical structures under certain conditions. Cells of A. tumefaciens strain C58 became spherical after incubation for 2 hr in the presence of Agrocin 84, a bacteriocin thought to alter the integrity of the bacterial cell wall (Smith and Hindley 1978). Pleomorphism is well documented for the closely related bacterial genus, Rhizobium (Tu and Braybrook 1981, Kaneshiro et al. 1983). Recently, sphere-shaped pleomorphs of the normally rod-shaped Azotobacter vinelandii also were found in soil (Lopez and Vela 1981).

The late time course experiments revealed the presence of microcolonies on the surface of the potato disc. The bacterial microcolonies were quite variable in size throughout the entire 12-day time course, and appeared to be initiated at different times during the 12-day period. This is consistent with the observation that both ACH5 and ACH5C3 colonize the disc at equivalent rates until well past the 5th to 6th day after inoculation, when tumors are visible. Each colony appeared to be surrounded by an electron-lucent coating. Although the exact nature of this coating is unknown, it is morphologically similar to polysaccharide material termed the glycocalyx (Costerton et al. 1981). This is of interest because the glycocalyx coating is thought to function as an ion exchange medium, possibly adsorbing materials such as antibiotics from the surrounding environment and affording the bacterium protection (Costerton et al. 1981). Tumor formation on pinto bean leaves is dramatically recuced when rifampin is introduced into the wound site up to 5 hr after

and Heberlein 1975). Sensitivity to rifampin is progressively diminished when more than 5 hr elapse between inoculation and antibiotic application. Microcolonies with glycocalyx-like coatings appear 4 to 8 hr after inoculation of potato discs. A similar phenomenon could explain the change in sensitivity of tumor formation on pinto bean leaves to rifampin.

Whereas microcolonies appeared to be anchored to the surface of potato tissue by the amorphous polysaccharide-like coating, small rod-like structures were observed bridging individual bacteria to the plant surface. If fixation artifacts were responsible, widespread occurrence of these structures would be expected. However, they were observed on a relatively small proportion of the bacteria on the surface. Patriquin et al. (1983) observed similar structures and suggested that they were responsible for anchoring Azospirillum spp. cells to sugarcane suspension culture cells. What role, if any, these rod-like structures play in bacterial adsorption or infection is not known.

CHAPTER SEVEN
SUMMARY

I characterized A. tumefaciens adsorption to potato tuber tissue that is competent to be transformed. It has been hypothesized from tumor formation studies that the adsorption of A. tumefaciens to plant tissue is specific and mediated by complementary molecular constituents, i.e. that o-antigenic region of the bacterial LPS 'recognizes' and binds to a specific galacturonan present in the plant cell wall. Subsequently, it has been shown by measuring adsorption directly that neither the LPS nor galacturonans of the plant mediate specific adsorption of A. tumefaciens to potato tuber tissue.

The purpose of my work was: i) to characterize the adsorption of A. tumefaciens to tissue that is competent for transformation (This was done by considering the bacterial suspension as a colloid and describing adsorption out of the liquid phase onto a solid in physical terms); ii) to provide a thermodynamic treatment of the adsorption data to distinguish between physical and chemical adsorption, and iii) to examine the adsorption sites, bacterial colonization, and general surface morphology during the infection process at the ultrastructural level.

The adsorption isotherm for A. tumefaciens was accurately defined by the Freundlich adsorption isotherm, that describes a system in which the available binding sites are not saturated. The adsorption isotherm was examined over a more limited initial inoculum density at three different temperatures (6°C,

28°C, and 38°C). Analysis of these isotherm data with the Clausius-Clapeyron equation shows that the heat of adsorption is close to zero. These combined data suggest that the adsorption of A. tumefaciens to potato tuber tissue is governed by physical processes.

Physical adsorption processes are partially governed by ionic van der Waals forces, and thus characterization of the ionic nature of the bacterial cell surface is important. In water the bacterium has an isoelectric point of 3, which indicates that the ratio of carboxyl or phosphate to amino groups of the bacterial cell surface is high.

If A. tumefaciens cells bind to potato tissues by physisorption, changes in the ionic or hydrophobic nature of the binding medium should alter bacterial adsorption. Optimum adsorption occurred at pH 7.2, and adsorption was reduced at either pH 4.5 or 9.5. Adsorption to hydrophobic plastic surfaces was insensitive to changes in pH. Conversely, the adsorption to hydrophilic plastic surfaces was optimal at pH 4.5 and decreased with increasing pH. Adsorption of A. tumefaciens cells to potato tissues was greatly reduced by detergents at concentrations as low as 0.05% (v/v). Anionic, cationic, and neutral detergents were found to be effective. The effect of agents that influence both hydrophobic and ionic forces is consistent with the proposed physisorption mechanism for A. tumefaciens.

The molecular components involved in A. tumefaciens adsorption to plant tissues are unknown. Proteolytic enzymes either enhanced or had no effect on adsorption. Hydrophobic pili participate in microbial attachment to several substrates and thus pili warrant further examination for their possible involvement in adsorption.

Surface polysaccharides would be likely candidates to possess ionizable groups, which would be affected by pH changes. Thus, these data do not rule out the possibility of the o-antigen reacting with the galacturonan material of the plant cell wall through some type of ionic interaction. However, it would seem unlikely that two acidic polysaccharides that possess a similar charge at a given pH will be bound to each other. It is more likely that each would react with a different molecular component possessing a more complementary charge. In conclusion, adsorption of A. tumefaciens to potato tuber tissue is a reversible, equilibrium, physisorption process described by the Freundlich adsorption isotherm.

LITERATURE CITED

Absolom, D. R., F. V. Lamberti, Z. Policova, W. Zingg, C. J. van Oss, and W. Neumann. 1983. Surface thermodynamics of bacterial adhesion. Appl. Environ. Microbiol. 46:90-97.

Anand, V. K., and G. T. Heberlein. 1975. Effect of rifampin application time on crown-gall tumor induction by Agrobacterium tumefaciens. Can. J. Bot. 53:2581-2588.

Arbuthnott, J. P., and C. J. Smyth. 1979. Bacterial adhesion in host-pathogen interactions in animals, pp. 165-198. in D. C. Ellwood, J. Melling, and P. Rutter (eds.), Adhesion of microorganisms to surfaces. Academic Press, New York.

Bal, A. K., S. Shantharam, and S. Ratnam. 1978. Ultrastructure of Rhizobium japonicum in relation to its attachment to root hairs. J. Bacteriol. 133:1393-1400.

Bauer, W. D. 1982. Attachment of Rhizobia to soybean roots. Plant Physiol. 69: Suppl. 143.

Beaud, G., P. Manigault, and G. Stoll. 1963. Observations sur des tumeurs vegetales incompletes. Plant Soil 47:25-37.

Beringer, J. E. 1980. The development of Rhizobium genetics. The fourth Fleming lecture. J. Gen. Microbiol. 116:1-7.

Berkeley, R. C. W., J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent. 1980. Microbial adhesion to surfaces. Ellis Harwood Ltd., Chichester, England. pp. 559.

Bhuvaneswari, T. V. 1981. Recognition mechanisms and infection process in legumes. Econ. Bot. 35:204-223.

Bhuvaneswari, T. V., A. A. Bhagwat, and W. D. Bauer. 1981. Transient susceptibility of root cells in four common legumes to nodulation by rhizobia. Plant Physiol. 68:1144-1149.

Bhuvaneswari, T. V., S. G. Pueppke, and W. D. Bauer. 1977. Role of lectins in plant-microorganism interactions. I. Binding of soybean lectin to rhizobia. Plant Physiol. 60:486-491.

Bitton, G., and K. C. Marshall. 1980. Adsorption of microorganisms to surfaces. John Wiley & Sons, New York, pp. 439.

Blakeman, J. P. 1982. Phylloplane interactions, pp. 308-333. in M. S. Mount and G. H. Lacy (eds.), Phytopathogenic prokaryotes, Vol. I. Academic Press, New York.

Bogers, R. J. 1972. On the interaction of Agrobacterium tumefaciens with cells of Kalanchoe diagremontiana, pp. 239-250. In H. P. Maas Gaesteranus (ed), Proc. 34rd. Int. Congr. Plant Pathog. Bact. Cent. Agric. Publ. & Doc., Wageningen, Netherlands.

Bohn, H. L., B. L. McNeal, and G. A. O'Conner. 1979. Soil Chemistry. John Wiley and Sons, New York, 329 pp.

Broughton, W. J. 1978. Control of specificity in legume- Rhizobium associations. J. Appl. Bacteriol. 45:165-194.

Broughton, W. J., A. W. S. M. van Egeraat, and T. A. Lie. 1980. Dynamics of Rhizobium competition for nodulation of Pisum sativum cv. Afghanistan. Can. J. Microbiol. 26:562-565.

Brown, N. A. 1923. Experiments with Paris daisy and rose to produce resistance to crown gall. Phytopathol. 13:87-99.

Brunauer, S. 1943. The adsorption of gases and vapors. Princeton University Press, Princeton, New Jersey. 511 pp.

Burton, A. J., and H. E. Carter. 1964. Purification and characterization of the lipid A component of the lipopolysaccharides from Escherichia coli. Biochem. 3:411-418.

Carlson, R. W., R. E. Sanders, C. A. Napoli, and P. Albersheim. 1978. Host-symbiont interactions, III. Purification and partial characterization of Rhizobium lipopolysaccharides. Plant Physiol. 62:912-917.

Chen, A. - P. T., and D. A. Phillips. 1976. Attachment of Rhizobium to legume roots as the basis for specific interactions. Physiol. Plant. 38:83-88.

Chilton, M. D., M. H. Drummond, D. J. Merlo, D. Sciaky, A. L. Montoya, M. P. Gordon, and E. W. Nester. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 3:263-271.

Conn, H. J. 1940. Biological stains. Biotech. Publ., Geneva, New York, 308 pp.

Cook, A. A., and R. E. Stall. 1977. Effect of watersoaking on response to Xanthomonas vesicatoria in pepper leaves. Phytopathol. 67:1101-1103.

Cooksey, D. A., and L. W. Moore. 1982. Biological control of crown gall with an agrocin mutant of Agrobacterium tumefaciens. Phytopathol. 72:919-921.

Costerton, J. W., R. T. Irvin, and K. J. Cheng. 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. 35:299-324.

Daniels, S. L. 1972. The adsorption of microorganisms onto surfaces: A review. Dev. Ind. Microbiol. 13:211-253.

Dazzo, F. B. 1980a. Adsorption of microorganisms to roots and other plant surfaces, pp. 253-316. In G. Bitton and K. C. Marshall (eds.), Adsorption of microorganisms to surfaces. Wiley-Interscience, New York.

Dazzo, F. B. 1980b. Microbial adhesion to plant surfaces, pp. 311-328. In R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (eds.), Microbial adhesion to surfaces. Ellis Horwood Ltd, Chichester, England.

Dazzo, F. B., and D. H. Hubbell. 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl. Microbiol. 30:1017-1033.

Dazzo, F. B., C. A. Napoli, and D. H. Hubbell. 1976. Adsorption of bacteria to roots as related to host specificity in the Rhizobium -clover symbiosis. Appl. Environ. Microbiol. 32:166-171.

De Cleene, M., and J. De Ley. 1976. The host range of crown gall. Bot. Rev. 42:389-466.

Deinema, M. H., and L. P. T. M. Zevenhuizen. 1971. Formation of cellulose fibrils by Gram-negative bacteria and their role in bacterial flocculation. Arch. Mikrobiol. 78:42-57.

Douglas, C. J., W. Halperin, and E. W. Nester. 1982. Agrobacterium tumefaciens mutants affected in attachment to plant cells. J. Bacteriol. 152:1265-1275.

Draper, J., I. A. Mackenzie, M. R. Davey, and J. P. Freeman. 1983. Attachment of Agrobacterium tumefaciens to mechanically isolated asparagus cells. Plant Sci. Lett. 29:227-236.

El-Kady, S., and S. Sule. 1981. Biological control of crown-gall tested on bean leaves. Acta Phytopathol. Acad. Sci. Hung. 16:307-313.

Farrand, S. K., C. I. Kado, and C. R. Ireland. 1981. Suppression of tumorigenicity by the IncW R plasmid pSa in Agrobacterium tumefaciens. Mol. Gen. Genet. 181:44-51.

Fletcher, M. 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23:1-6.

Fletcher, M., M. J. Latham, J. M. Lynch, and P. R. Rutter. 1980. The characteristics of interfaces and their role in microbial attachment, pp. 67-78. in R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (eds.), Microbial adhesion to surfaces. Ellis Harwood Ltd. Chichester, England.

Fred, E. B., I. L. Baldwin, and E. McCoy. 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Press, Madison, 788pp.

Foster, R. C., and G. D. Bowen. 1982. Plant surfaces and bacterial growth: the rhizosphere and rhizoplane, pp. 159-185. In M. S. Mount and G. H. Lacy (eds.), Phytopathogenic prokaryotes, Vol. 1. Academic Press, New York.

Galanos, C., and O. Luderitz. 1975. Electrodialysis of lipopolysaccharides and their conversion to uniform salt forms. Eur. J. Biochem. 54:603-610.

Gamborg, O. L., and L. R. Wetter. 1975. Plant tissue culture methods. Nat. Res. Couns. Canada, Saskatoon, Saskatchewan. 110 pp.

Gibbons, R. J., I. Etherden, and Z. Skobe. 1983. Association of fimbriae with the hydrophobicity of Streptococcus sanguis FC-1 and adherence to salivary pellicles. Infect. Immun. 41:414-417.

Glogowsky, W., and A. G. Galsky. 1978. Agrobacterium tumefaciens site attachment as a necessary prerequisite for crown gall tumor formation on potato discs. Plant Physiol. 61:1031-1033.

Goodman, R. N., P.-Y. Huang, and J. A. White. 1976. Ultrastructural evidence for immobilization of an incompatible bacterium, Pseudomonas pisi, in tobacco leaf tissue. Phytopathol. 66:754-764.

Goodman, R. N., and S. B. Plurad. 1971. Ultrastructural changes in tobacco undergoing the hypersensitive reaction caused by plant pathogenic bacteria. Physiol. Plant Pathol. 1:11-15.

Goodman, R. N., D. J. Politis, and J. A. White. 1977. Ultrastructural evidence of an 'active' immobilization process of incompatible bacteria in tobacco leaf tissue: A resistance reaction, pp. 423-437. In B. Solheim, and J. Raa, (eds.), Cell wall biochemistry related to specificity in host-plant pathogen interactions. Universitetsforlaget, Oslo, Norway.

Gordon, A., S., and F. J. Millero. 1984. Electrolyte effects on attachment of an estuarine bacterium. Appl. Environ. Microbiol. 47:495-499.

Gotz, E. -M. 1980. Attachment to plant root surface and nitrogenase activity of Rhizobia associated with Petunia plants. Z. Pflanzenphysiol. 98:464-470.

Gotz, E. M., and D. Hess. 1980. Nitrogenase activity induced by wheat plants. Z. Pflanzenphysiol. 98:453-458.

Gurley, W. B., J. D. Kemp, M. J. Albert, D. W. Sutton, and J. Callis. 1979. Transcription of Ti plasmid-derived sequences in three octopine-type crown gall tumor lines. Proc. Nat. Acad. Sci. U.S.A. 76:2828-2832.

Haas, J. H., and J. Rotem. 1976. Pseudomonas lachrymans adsorption, survival, and infectivity following precision inoculation of leaves. Phytopathol. 66:992-997.

Hanold, D. 1983. In vitro transformation of protoplast-derived Hyoscyamus muticus cells by Agrobacterium tumefaciens. Plant Sci. Lett. 30:177-183.

Harden, V. P., and J. O. Harris. 1953. The isoelectric point of bacterial cells. J. Bacteriol. 65:198-202.

Hardy, R. W. F., and U. D. Havelka. 1975. Nitrogen fixation research a key to world food. Science 188:633-643.

Hess, D., E. -M. Gotz, M. Harfold. 1982. Rhizobium sp. 32H1 associated with wheat and petunia: invasion of plant root tissue. Z. Pflanzenphysiol. 107:81-84.

Hildebrand, E. M. 1942. A micrurgical study of crown gall infection in tomato. Jour. Agric. Res. 65:45-59.

Higashi, S., and M. Abe. 1980. Scanning electron microscopy of Rhizobium trifolii infection sites on root hairs of white clover. Appl. Environ. Microbiol. 40:1094-1099.

Hobbs, S. L. A., and J. D. Matton. 1983. Variability and interaction in the Pisum sativum L. - Rhizobium leguminosarum symbiosis. Can. J. Plant Sci. 63:591-599.

Holl, F. B., and T. A. La Rue. 1976. Genetics of legume plant hosts. pp. 391-399. In W. E. Newton and C. J. Nyman (eds.), Proc. Int. Sym. on Nitrogen Fixation. Vol. II. Washington State Univ. Press, Pullman, Washington.

Holmes, F. O. 1929. Local lesions in tobacco mosaic. Bot. Gaz. 87:39-55.

Jansen van Rensburg, H. J., and B. A. Strijdom. 1982. Root surface association in relation to nodulation of Medicago sativa. Appl. Environ. Microbiol. 44:93-97.

Johnson, K. G., and M. B. Perry. 1976. Improved techniques for the preparation of bacterial lipopolysaccharides. Can. J. Microbiol. 22:29-34.

Kaneshiro, T., F. L. Baker, and D. E. Johnson. 1983. Pleomorphism and acetylene-reducing activity of free-living rhizobia. J. Bacteriol. 153:1045-1050.

Kerr, A. 1975. A genetic model for pathogenicity in Agrobacterium and for tumor induction in plants. J. Theor. Biol. 51:409-417.

Kiraly, Z. 1980. Defenses triggered by the invader: hypersensitivity, pp. 201-224. In J. G. Horsfall, and E. B. Cowling (eds.), Plant disease an advanced treatise, Volume 5. Academic Press, New York.

Klement, Z. 1963. Rapid detection of the pathogencity of phytopathogenic pseudomonads. Nature 199:299-300.

Klement, Z. 1977. Cell contact recognition versus toxin action in induction of bacterial hypersensitive reaction. Acta Phytopathol. Acad. Sci. Hung. 6:115-118.

Klement, Z. 1982. Hypersensitivity, pp. 149-177. In M. S. Mount, and G. H. Lacy, (eds.), The phytopathogenic prokaryotes, Volume 2. Academic Press, New York.

Klement, Z., G. L. Farkas, and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathol. 54:474-477.

Klement, Z., and R. N. Goodman. 1967. The role of the living bacterial cell and induction time in the hypersensitive reaction of the tobacco plant. Phytopathol. 57:322-323.

Klement, Z., and L. Lovrekovich. 1961. Defense reactions induced by phytopathogenic bacteria in bean pods. Phytopathol. Z. 41:217-227.

Klement, Z., and L. Lovrekovich. 1962. Studies on host-parasite relations in bean pods infected with bacteria. Phytopathol. Z. 45:81-88.

Krens, F. A., L. Molendijk, G. J. Wullems, and R. A. Schilperoort. 1982. In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature 296:72-74.

Larrimore, S., H. Murchison, T. Shiota, S. M. Michalek, and R. Curtiss III. 1983. In vitro and in vivo complementation of Streptococcus mutans mutants defective in adherence. Inf. Immun. 42:558-566.

Law, I. J., A. J. Mort, and W. D. Bauer. 1982. Nodulation of soybean by Rhizobium japonicum mutants with altered capsule synthesis. Planta 154:100-109.

Leben, C., and R. E. Whitmoyer. 1979. Adherence of bacteria to leaves. Can. J. Microbiol. 25:896-901.

Lie, T. A., P. C. J. M. Timmermans, and G. Ladzinsky. 1981. Host controlled nitrogen fixation in Pisum sativum ecotype fulvum, p. 419. In A. H. Gibson and W. E. Newton (eds.), Current perspectives in nitrogen fixation. Aust. Acad. of Sci., Canberra, Australia.

Lippincott, B. B., and J. A. Lippincott. 1969. Bacterial attachment to a specific wound site as an essential stage in tumor initiation by Agrobacterium tumefaciens. J. Bacteriol. 97:620-628.

Lippincott, B. B., J. B. Margot, and J. A. Lippincott. 1977a. Plasmid content and tumor initiation complementation by Agrobacterium tumefaciens IIBNV6. J. Bacteriol. 132:824-831.

Lippincott, B. B., M. H. Whatley, and J. A. Lippincott. 1977b. Tumor induction by Agrobacterium involves attachment of the bacterium to a site on the host plant cell wall. Plant Physiol. 59:388-390.

Lippincott, J. A., C. C. Chang, V. R. Creaser-Pence, P. R. Birnberg, S. S. Rao, J. B. Margot, M. H. Whatley, and B. B. Lippincott. 1978. Genetic determinants governing enhancement of tumor initiation by avirulent agrobacteria, Agrobacterium-host adherence and octopine synthesis, pp. 189-197. In Proc. 4th Int. Conf. Plant. Path. Bact. Gibert-Clarey, Tours, France

Lippincott, J. A., and G. T. Heberlein. 1965a. The induction of leaf tumors by Agrobacterium tumefaciens. Am. J. Bot. 52:396-403.

Lippincott, J. A., and G. T. Heberlein. 1965b. The quantitative determination of the infectivity of Agrobacterium tumefaciens. Am. J. Bot. 52:856-863.

Lippincott, J. A., and B. B. Lippincott. 1970. Enhanced tumor initiation by mixtures of tumorigenic and nontumorigenic strains of Agrobacterium. Inf. Immun. 2:623-630.

Lippincott, J. A., and B. B. Lippincott. 1975. The genus Agrobacterium and plant tumorigenesis. Annu. Rev. Microbiol. 29:377-405.

Lippincott, J. A., and B. B. Lippincott. 1977. Nature and specificity of the bacterium-host attachment in Agrobacterium infection, pp. 438-451. In B. Solheim and J. Raa (eds.), Cell wall biochemistry related to specificity in host-plant pathogen interaction. Universitetsforlaget, Oslo, Norway.

Lippincott, J. A., and B. B. Lippincott. 1978a. Cell walls of crown-gall tumors and embryonic plant tissues lack Agrobacterium adherence sites. Science 199:1075-1077.

Lippincott, J. A., and B. B. Lippincott. 1978b. Tumor initiation complementation on bean leaves by mixtures of tumorigenic and nontumorigenic Agrobacterium rhizogenes. Phytopathol. 68:365-370.

Lippincott, J. A., and B. B. Lippincott. 1980. Microbial adherence in plants, pp. 377-397. In W. H. Beachey (ed.), Bacterial adherence. Chapman and Hall, London, England.

Lippincott, J. A., and B. B. Lippincott. 1983. Adherence and host recognition in Agrobacterium infection. Third Int. Symp. Microbiol. Ecol., Michigan (abstr.).

Lips, A., and N. E. Jessup, 1979. Colloidal aspects of bacterial adhesion, pp. 5-27. In D. C. Ellwood, J. Melling, and R. Rutter (eds.) Adhesion of microorganisms to surfaces. Academic Press, New York.

Ljunggren, H. 1961. Transfer of virulence in Rhizobium trifolii. Nature 191:623.

Lopez, J. G., and G. R. Vela. 1981. True morphology of the Azotobacteraceae-filterable bacteria. Nature 289:588-590.

Luderitz, O., K. Jann, and R. Wheat. 1968. Somatic and capsular antigens of Gram-negative bacteria. Compr. Biochem. 26A:105-228.

Maier, R. J., and W. J. Brill. 1976. Ineffective and non-nodulating mutant strains of Rhizobium japonicum. J. Bacteriol. 127:763-769.

Marshall, K. C. 1976. Interfaces in microbial ecology. Harvard Univ. Press, Cambridge, Massachusetts. 156 pp.

Marshall, K. C., and R. H. Cruickshank. 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Arch. Microbiol. 91:29-40.

Marshall, K. C., R. H. Cruickshank, and H. V. A. Bushley. 1975. The orientation of certain root-nodule bacteria at interfaces, including legume root-hair surfaces. J. Gen. Microbiol. 91:198-200.

Marton, L., G. L. Wullems, L. Molendijk, and R. A. Schilperoort. 1979. In-vitro transformation of cultured cells from Nicotiana tabacum by Agrobacterium tumefaciens. Nature 277:129-131.

Matthysse, A. G. 1983. Role of bacterial cellulose fibrils in Agrobacterium tumefaciens infection. J. Bacteriol. 154:906-915.

Matthysse, A. G., and R. H. G. Gurlitz. 1982. Plant cell range for attachment of Agrobacterium tumefaciens to tissue culture cells. Physiol. Plant Pathol. 21:381-387.

Matthysse, A. G., K. V. Holmes, and R. H. G. Gurlitz. 1981. Elaboration of cellulose fibrils by Agrobacterium tumefaciens during attachment to carrot cells. J. Bacteriol. 145:583-995.

Matthysse, A. G., V. K. Holmes, and R. H. G. Gurlitz. 1982. Binding of Agrobacterium tumefaciens to carrot protoplasts. Physiol. Plant Pathol. 20:27-33.

Matthysse, A. G., P. M. Wyman, and K. V. Holmes. 1978. Plasmid-dependent attachment of Agrobacterium tumefaciens to plant tissue culture cells. Inf. Immun. 22:516-522.

Menzel, G., H. Uhlig, and G. Weichsel. 1972. Uber die besiedlung der wurzeln einiger leguminosen and nichtleguminosen mit rhizobien and anderen bodenbakterien. Zentralbl. Bakteriol., Parasitenk., D. Infektionskr. Hyg. Abt. Microbiol. Landwirtsch. Tech. Mikrobiol. 127-248-358.

Merlo, D. S. 1978. Crown gall--A unique disease, pp. 201-213. In J. G. Horsfall and E. B. Cowling (eds.) Plant disease an advanced treatise, Vol III. Academic Press, New York.

Meyer, M. C., and S. G. Pueppke. 1980. Differentiation of Rhizobium japonicum strain derivatives by antibiotic sensitivity patterns, lectin binding, and utilization of biochemicals. Can. J. Microbiol. 26:606-617.

Moore, L. W., and D. A. Cooksey. 1981. Biology of Agrobacterium tumefaciens: Plant interactions, pp. 15-46. In K. L. Giles and A. G. Atherly (eds.), Int. Rev. Cyt. Suppl. 13. Academic Press, New York.

Muller, K. O. 1959. Hypersensitivity, pp. 469-519. In J. G. Horsfall, and A. E. Dimond (eds.), Plant pathology an advanced treatise. Academic Press, New York.

Napoli, C. A., F. Dazzo, and D. Hubbell. 1975. Production of cellulose microfibrils by Rhizobium. Appl. Microbiol. 30:123-131.

Nester, E. W., and T. Kosuge. 1981. Plasmids specifying plant hyperplasias. Annu. Rev. Microbiol. 35:531-564.

New, P. B., J. J. Scott, C. R. Ireland, S. K. Farrand, B. B. Lippincott, and J. A. Lippincott. 1983. Plasmid pSa causes loss of LPS-mediated adherence in Agrobacterium. J. Gen. Microbiol. 129:3657-3660.

Nissen, P. 1971. Choline sulfate permease: transfer of information from bacteria to higher plants II. Induction processes, pp. 201-212. In L. Ledoux (ed.), Information molecules in biological systems. North Holland Publ. Co. Amsterdam, Netherlands.

Norkrans, B. 1981. The hydrophobicity of bacteria - an important factor in their adhesion at the air-water interface. Arch. Microbiol. 128:267-270.

Nutman, P. S. 1981. Hereditary host factors affecting nodulation and nitrogen fixation, pp. 194-204. In A. H. Gibson and W. E. Newton (eds.), Current perspectives in nitrogen fixation, Proc. 4th Int. Sym. Nitrogen Fixation. Australian Acad. Sci., Canberra, Australia.

Ofek, I., and E. H. Beachey. 1980. Bacterial adherence. Adv. Int. Med. 25:503-532.

Ohyama, K., L. E. Pelcher, and A. Schaefer. 1979. In vitro binding of Agrobacterium tumefaciens to plant cells from suspension culture. Plant Physiol. 63:382-387.

Otto, J. C. G. 1975. Ecology, physiology and genetics of fimbrae and pili. Ann. Rev. Microbiol. 29:79-108.

Patriquin, D. G., J. Dobereiner, and D. K. Jain. 1983. Sites and processes of association between diazotrophs and grasses. Can J. Microbiol. 29:900-915.

Perkins, A. T. 1925. The effect of bacterial numbers on the nodulation of Virginia soybeans. J. Agric. Res. 30:95-96.

Pueppke, S. G. 1984a. Adsorption of bacteria to plant surfaces. In T. Kosuge and E. W. Nester (eds.), Plant-microbe interactions, molecular and genetic perspectives. Macmillan Publishing Co., New York (in press).

Pueppke, S. G. 1984b. Adsorption of slow - and fast-growing Rhizobia to soybean and cowpea roots. Plant Physiol. (in press).

Pueppke, S. G., and U. K. Benny. 1981. Induction of tumors on Solanum tuberosum L. by Agrobacterium: quantitative analysis, inhibition by carbohydrates, and virulence of selected strains. Physiol. Plant Pathol. 18:169-179.

Pueppke, S. G., and U. K. Benny. 1983. Agrobacterium tumefaciens in potato: effect of added Agrobacterium lipopolysaccharides and the degree of methylation of added plant galacturonans. Physiol. Plant Pathol. 23:439-446.

Pueppke, S. G., and U. K. Benny. 1984. Adsorption of tumorigenic Agrobacterium tumefaciens cells to susceptible potato tuber tissue. Can. J. Microbiol. 30: (in press).

Rao, S. S., B. B. Lippincott, and J. A. Lippincott. 1982. Agrobacterium adherence involves the pectic portion of the host cell wall and is sensitive to the degree of pectin methylation. Physiol. Plant Pathol. 56:374-380.

Reporter, M., D. Raveed, and G. Norris. 1975. Binding of Rhizobium japonicum to cultured soybean root cells: morphological evidence. Plant Sci. Lett. 5:73-76.

Roberts, W. P., and A. Kerr. 1974. Crown gall induction: serological reactions, isozyme patterns, sensitivity to mitomycin C and to bacteriocin, of pathogenic and non-pathogenic strains of Agrobacterium radiobacter. Physiol. Plant Pathol. 4:81-91.

Rosenberg, M. 1981. Bacterial adherence to polystyrene, a replica method of screening for bacterial hydrophobicity. Appl. Environ. Microbiol. 42:375-377.

Rosenberg, M., S. Rottem, and E. Rosenberg. 1982. Cell surface hydrophobicity of smooth and rough Proteus mirabilis strains as determined by adherence to hydrocarbons. FEMS Microbiol. Lett. 13:167-169.

Rosenberg, E., A. Gottlieb, and M. Rosenberg. 1983. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by Emulsan. Inf. Immun. 39:1024-1028.

Sahlman, K., and G. Fahraeus. 1963. An electron microscope study of root-hair infection by Rhizobium. J. Gen. Microbiol. 33:425-427.

Saunders, L. 1971. Principles of physical chemistry for biology and pharmacy. Oxford Univ. Press, London, England. 420 pp.

Schilperoort, R. A. 1969. Investigations on plant tumors--crown gall. PhD. Thesis, Univ. of Leiden, Netherlands.

Schmidt, E. L. 1979. Initiation of plant root-microbe interactions. Annu. Rev. Microbiol. 33:355-376.

Sequeira, L. 1980. Defenses triggered by the invader: recognition and compatibility phenomena, pp. 179-200. In J. G. Horsfall, and E. B. Cowling, (eds.), Plant disease an advanced treatise, Volume 5. Academic Press, New York.

Sequeira, L., G. Gaard, and G. A. DeZoeten. 1977. Attachment of bacteria to host cells walls: Its relation to mechanisms of induced resistance. Physiol. Plant Pathol. 10:43-50.

Sequeira, L, and T. L. Graham. 1977. Agglutination of avirulent strains of Pseudomonas solanacearum by potato lectin. Physiol. Plant Pathol. 11:43-54.

Shimshick, E. J. and R. R. Hebert. 1978. Adsorption of Rhizobia to cereal roots. Biochem. Biophys. Res. Comm. 84:736-742.

Shimshick, E. J. and R. R. Hebert. 1979. Binding characteristics of N_2-fixing bacteria to cereal roots. Appl. Env. Microbiol. 38:447-453.

Sigee, D. C., V. A. Smith, and J. Hindley. 1982. Passage of bacterial DNA into host cells during in vitro transformation of Nicotiana tabacum by Agrobacterium tumefaciens. Microbios 34:113-132.

Sing, V. O., and M. N. Schroth. 1977. Bacteria-plant cell surface interaction: Active immobilization of saprophytic bacteria in plant leaves. Science 197:759-761.

Slusarenko, A. J., and R. K. S. Wood. 1983. Agglutination of Pseudomonas phaseolicola by pectic polysaccharide from leaves of Phaseolus vulgaris Physiol. Plant Pathol. 23:217-227.

Smith, V. A., and J. Hindley. 1978. Effect of agrocin 84 on attachment of Agrobacterium tumefaciens to cultured tobacco cells. Nature 276:498-500.

Solheim, B. 1983. Possible role of lectins in binding rhizobia to host roots, pp. 539-547. In T. C. Bog-Hansen and G. Spengler (eds.), Lectins-Biology, biochemistry, clinical biochemistry, Volume 3. Walter de Gruyter, Berlin, Germany.

Solheim, B., and J. Paxton. 1981. Recognition in Rhizobium-legume systems, pp. 71-83. In R. C. Staples and G. H. Toenniessen (eds.), Plant disease control: Resistance and susceptibility. John Wiley, New York.

Spiess, L. D., B. B. Lippincott, and J. A. Lippincott. 1971. Development and gametophore induction in the moss Pylaisiella selwynii as influenced by Agrobacterium tumefaciens. Am. J. Bot. 58:726-731.

Spiess, L. D., B. B. Lippincott, and J. A. Lippincott. 1976. The requirement of physical contact for moss gametophore induction by Agrobacterium tumefaciens. Am. J. Bot. 58:726-731.

Spiess, L. D., B. B. Lippincott, and J. A. Lippincott. 1977a. Comparative response of Pylaisiella selwynii to Agrobacterium and Rhizobium species. Bot. Gaz. 138:35-40.

Spiess, L. D., J. C. Turner, P. G. Mahlberg, B. B. Lippincott, and J. A. Lippincott. 1977b. Adherence of Agrobacteria to moss protonema and gametophores viewed by scanning electron microscopy. Am. J. Bot. 64:1200-1204.

Stacey, G., A. S. Paau, and W. J. Brill. 1980. Host recognition in the Rhizobium-soybean symbiosis. Plant Physiol. 66:609-614.

Stacey, G., A. S. Paau, K. D. Noel, R. J. Maier, L. E. Silver, and W. J. Brill. 1982. Mutants of Rhizobium japonicum defective in nodulation. Arch. Microbiol. 132:219-224.

Stall, R. E., and A. A. Cook. 1979. Evidence that bacterial contact with the plant cell is necessary for the hypersensitive reaction but not the susceptible reaction. Physiol. Plant Pathol. 14:77-84.

Stonier, T. 1956. Radioautographic evidence for the intercellular location of crown gall bacteria. Am. J. Bot. 43:647-655.

Suslow, T. V. 1982. Role of root-colonizing bacteria in plant growth, pp. 187-223. In M. S. Mount, and G. H. Lacy (eds.), Phytopathogenic prokaryotes, Volume 1. Academic Press, New York.

Thomashow, M. F., R. Nutter, A. L. Montoya, M. P. Gordon, and E. W. Nester. 1980. Integration and organization of Ti plasmid sequences in crown gall tumors. Cell 19:729-739.

Tompkins, F. C. 1978. Chemisorption of gases and metals. Academic Press, New York, 250 pp.

Tu, J. C., and G. Braybrook, 1981. Structural organization of the rhizobial root nodule of clover. Microbios 32:77-88.

Turakhia, M. H., K. E. Cooksey, and W. G. Characklis. 1983. Influence of a calcium-specific chelant on biofilm removal. Appl. Environ. Microbiol. 46:1236-1238.

Turgeon, B. G., and W. D. Bauer. 1982. Early events in the infection of soybean by Rhizobium japonicum. Time course and cytology of the initial infection process. Can J. Bot. 60:152-161.

Vincent, J. M. 1980. Factors controlling the legume- Rhizobium symbiosis, pp. 103-129. In W. E. Newton and W. H. Orme-Johnson (eds.), Nitrogen fixation II. Univ. Park Press, Baltimore.

Ward, H. M. 1902. On the relations between host and parasite in the Bromes and their brown rust, Puccinia dispersa (Erikss.). Ann. Bot. 16:233-315.

Watson, B., T. C. Currier, M. P. Gordon, M. D. Chilton, and E. W. Nester. 1975. Plasmid required for virulence of Agrobacterium tumefaciens. J. Bacteriol. 123:255-264.

Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure, pp 83-91. In R. L. Whistler (ed.), Methods in carbohydrate chemistry, Vol. 5. Academic Press, New York.

Whatley, M. H., J. S. Bodwin, B. B. Lippincott, and J. A. Lippincott. 1976. Role of Agrobacterium cell envelope lipopolysaccharide in infection site attachment. Inf. Immun. 13:1080-1083.

Whatley, M. H., J. B. Margot, J. Schell, B. B. Lippincott, and J. A. Lippincott. 1978. Plasmid and chromosomal determination of Agrobacterium adherence specificity. J. Gen. Microbiol. 107:395-398.

Whatley, M. H., and L. D. Spiess. 1977. Role of bacterial lipopolysaccharides in attachment of Agrobacterium to moss. Plant Physiol. 60:765-766.

Wullums, G. J., L. Molendijk, G. Ooms, and R. A. Schilperoort. 1981. Differential expression of crown gall tumor markers in transformants obtained after in vitro Agrobacterium-induced transformation of cell wall regenerating protoplasts derived from Nicotiana tabacum. Proc. Nat. Acad. Sci. USA 78:4344-4348.

Yadau, N. S., K. Postle, R. K. Saiki, M. F. Thomashow, and M. D. Chilton. 1980. T-DNA of a crown gall teratoma is covalently joined to the host plant DNA. Nature 287:458-461.

Zarr, J. H. 1974. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, New Jersey. 620 pp.

Zurkowski, W. 1980. Specific adsorption of bacteria to clover root hairs, related to the presence of the plasmid pWZ2 in cells of Rhizobium trifolii. Microbios 27:27-32.

BIOGRAPHICAL SKETCH

Daniel Albert Kluepfel was born in St. Louis, Missouri on January 29, 1955, to Marion and Albert Kluepfel. He attended school in the St. Louis area till his family moved to Jefferson County, Missouri. Upon completion of high school in 1973 he took a position as a computer-programmer operator for three years. During this time he also enrolled in the University of Missouri at St. Louis, from which he received his Bachelor of Arts in biology December of 1978. He came to the University of Florida in the fall of 1979. He received his Ph.D. in plant pathology with a minor in microbiology in 1984. While a graduate student in Florida Dan married Marjan van Nuffelen. In March of 1984 he was awarded a Fulbright fellowship to do research at the Agricultural University of Wageningen in the Netherlands upon completion of his Ph.D.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Steven G. Pueppke, Chairman
Associate Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert E. Stall
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David J. Mitchell
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William B. Gurley
Assistant Professor of
Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Brij M. Moudgil
Associate Professor of Material Science

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1984

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School

UNIVERSITY OF FLORIDA
3 1262 08553 2058